Establishment and characterization of cell clones from the Papilio cell line RIRI-PaDe-3 by a high-efficiency clonal method

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Abstract Cell cloning is of great importance in keeping particular properties of cultured cells, and interesting cells can be selected by cloning from heterogeneous cell populations. In addition, continuous cell lines usually from primary culture are prone to heterologous constitution and genetic instability, so that supplementary cloning steps are necessary for achieving a homogenous cell population. In this study, limiting dilution culture and feeder layer culture were originally used for cloning RIRI-PaDe-3 cell line, but both failed. Afterward, we designed a cloning protocol

which was composed of two steps: cells in semisolid medium with seeding density in the range of 3.05×10^5 – 6.10×10^5 cells/mL formed colonies from monodispersed cell suspensions; 40 well-dispersed colonies were removed from the suspended state by using micromanipulator system and finally scaled up. To determine whether this method can isolate cell lines possessing characteristics different from the parent population, we made an evaluation of cells monoclonal in biological characteristics. Significant differences have been found among clones isolated from the RIRI-PaDe-3 insect cell line in cell morphology, chromosome numbers, and genetic background. Thus the indicated modified semisolid medium cloning protocol was advantageous to the convenient and genuine cloning from the previously heterogeneous population.

Keywords Cell cloning · Semisolid culture · Micromanipulation · Inter-simple sequence repeat

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Introduction

The insect cell-baculovirus expression vector system (IC-BEVS) is a eukaryotic expression system with a fully functional post-translational modification system that has been developed rapidly in recent years. Due to its high safety and large carrying capacity, this system

can be used to efficiently express exogenous genes (Hu 2005; Sokolenko et al. 2012). Its application is very valuable in a variety of fields including genetic engineering, protein engineering, vaccine engineering, development of drugs, and biopesticides (Ikonomou et al. 2003; Swiech et al. 2012; Yamaji et al. 2008). Insect cell lines are normally obtained by cutting the whole insect bodies or tissues into small pieces via mechanical force and culturing for some time. As a result, the cell lines are derived from different tissues, which give rise to remarkable differences in cell morphology and viral susceptibility (Pan et al. 2010; Wen et al. 2009). It is necessary to isolate the cells from heterogeneous cell populations. The most commonly used host cell lines for protein expression include the *Spodoptera frugiperda* Sf9 and *Trichoplusia ni* High Five cell lines, which were obtained by cloning. The Sf-9 cell line was derived from the Sf21 cell line, but it is superior to the latter in terms of environmental adaptability and biological properties. Similarly, the High Five cell line was derived from the Tn-5 cell line and demonstrated higher cell growth density, growth rate, and protein production compared with the Tn-5 (Drugmand et al. 2012). Hence, selecting pure clones from heterogeneous cell populations can produce clones that display significant differences in yields of various biological products (Lindgren et al. 2009; Nguyen et al. 2011).

Single cell cloning is a laboratory routine to isolate and keep particular properties of cultured cells. There are various common methods used for single cell cloning: limiting dilution, feeder layer culture, and semisolid cloning (Allieri et al. 1990; Llames et al. 2015). Limiting dilution cloning is a simple, gentle, and relatively cost-efficient process to obtain single cells with reasonable throughput (Browne and Al-Rubeai 2007). However, due to the statistical nature of the process and the lack of control over an individual cell, there is no guarantee that the colonies arose from single cells (Gross et al. 2015). Feeder layer cells are used as a substratum to condition the medium on which cells, particularly at low cell density, are grown (Llames et al. 2015). For cloning, feeder layer cells should be used from the same cell line. Depending on cells, the feeder cells can be placed either inside the insert, with the cells of interest in the multiwell plate, or in the opposite way (Lynn 2001). The method of semisolid cloning is developed to bypass some of the major bottlenecks and challenges of the limiting

dilution cloning process by eliminating the sub cloning steps (Hou et al. 2014). Semisolid cloning is the process by which transfected or fused cells are suspended in a semisolid medium, and the cells become immobilized (Mcintosh and Rehtoris 1974). They then can create distinct colonies, which can be picked and isolated (Dharshanan et al. 2011). Compared to the two above mentioned cloning techniques, it can be done faster and efficiently in cloning mammalian cells using semisolid cloning. However, it is seldom used in cloning insect cells. The main reason is that insect cells grow very slowly at low densities because of the demand for autocrine growth factors, which accumulate to sufficient levels only at high cell densities (Kioukia et al. 1995). On the other hand, it is hard to form single, well isolated colonies at high cell densities. As technology advances, automated cell picking systems are increasingly used for accelerating cell line development processes. For example, Nguyen et al. have proven that for insect cell lines, the use of an automated clone picking system such as ClonePix can both shorten the time and increase the survival rate from 1 to 10% using a serial dilution method, to more than 75% (Nguyen et al. 2011). However, it cannot serve as a routine method for cell research due to its high cost and limited application scope. Therefore, based on the existing methods to develop a simple and high-efficiency technique for insect single-cell cloning is of important significance for research on the basic cell biology of insects.

Previous research has found that compared with the Sf-9 and High Five cell lines, RIRI-PaDe-3 cells, which were developed from neonate larvae of *Papilio demoleus* (Lepidoptera: Papilionidae), can express a higher level of recombinant secreted alkaline phosphatase (SEAP) and have a longer expression period for exogenous proteins (Ding et al. 2015). Distinct morphological differences within the Papilio cell line RIRI-PaDe-3 population have been observed, with round, epithelial-like and spindle-shaped cells coexisting. We also found that the viral susceptibility of wild-type *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) within the polyclonal cell population is heterogeneous (unpublish). Therefore, we wanted to design an easy cloning protocol that will be readily applicable to authentic monoclonal culture derived from RIRI-PaDe-3 for improving recombinant protein expression levels in future

studies. In the present research, modified semisolid cloning protocol was designed to isolate and culture single cells ascertained under micromanipulator system. In addition, for comparison purposes, two cell cloning technologies: limiting dilution and feeder layer culture, were used as parallel group trial to compare the performance of the new protocol. A variety of clones was isolated from the heterogeneous RIRI-PaDe-3 insect cell population, that were compared in their morphology, karyotype and genetics background in order to determine that cloning can lead to cell lines possessing characteristics different from the parent population.

Materials and methods

Cell line

The Papilio cell line RIRI-PaDe-3 was established by Ding et al. (2015) and preserved in Research Institute of Resource Insects (Kunming, China). RIRI-PaDe-3 is an adherent cell line and is cultured in an incubator (Binder, Tuttlingen, Germany, Cat. 9020-1036 KB240) at 25 °C, using Modified Grace insect cell culture medium (Gibco, Waltham, MA, USA, Cat. 932747) (Zhang et al. 2011) with 20% fetal bovine serum (FBS) (Biological industries, Beit Haemek, Israel, Cat. 1529263).

Cloning

Limiting dilution cloning

Using the protocols established by Freshney (2004), at the exponential growth phase, RIRI-PaDe-3 cells were diluted with conditioned medium to a concentration of 2×10^4 cells/mL. The conditioned medium was obtained from cells in the exponential growth phase and was supplemented with 10% FBS, and added to 96-well plates (Corning, Corning, NY, USA, Cat. 3997) (100 µL/well) except for well A1 which was left empty. The 200 µL of diluted cell suspension was added to well A1. Then, a single channel pipettor (Brand, Inc, Wertheim, Germany) was used to transfer 100 µL of suspended cells from the first well to well B1 quickly followed by gentle mixing. Repeating this operation, the cells were diluted from well B1 through H1. To make the entire column (well A1 through H1)

with the same volume, 100 µL of diluted cell suspension from well H1 was discarded. The 8-channel micropipettor (Eppendorf, Inc, Hamburg, Germany) was used to add 100 µL of conditioned medium to each well in column 1 and transfer 100 µL from the wells in the first column (A1 through H1) to those in second column (A2 through H2) quickly followed by gentle mixing via pipetting. These dilutions were repeated until the entire plate was across. The plate was incubated at 25 °C. The wells that contained just a single cell were identified by microscope after 4 days and marked. Any cell population arising from one of these marked wells was considered a clonal population and subcultured from the wells into larger vessels.

Cloning with a feeder layer

The cell suspension was diluted and dispensed into a small petri dish (Corning, Cat. 430166) so as to make a cell density of 1 cell/microscope field using a 10×20 objective and ocular lens combination. For the isolation of single cells, a commercial micromanipulator (Eppendorf, Model 5188), equipped with oil pressure injector (Eppendorf, Model 5176) and twintip-holder (Eppendorf, Model 5188), mounted onto an inverse phase contrast microscope (Nikon, Minato-ku, Tokyo, Japan, eclipse TS100) was used according to the manufacturer's instructions. All the equipment was placed in a laminar flow cabinet (Airtech, model SW-CJ-2FD). Eppendorf VacuTip (Cat. 930001015) was used to pick out selected cell. The isolated cell was released into a well of a 24-well culture plate filled with a small amount of medium. Hanging cell culture inserts (Millipore, Burlington, MA, USA, model PIHT12R48) were placed into each well. The RIRI-PaDe-3 whole cells were selected to be used for the feeder culture and distributed into each cell culture

Fig. 1 Schematic presenting the configuration of feeder cell layers supporting culture of single cells

insert (Fig. 1). The plate was incubated at 25 °C. Any cell population arising was considered a clonal population and subcultured from the wells into larger vessels.

Cloning with modified semisolid medium protocol

Cells were collected from exponential phase at 3 days post inoculation and diluted to 1.22×10^6 , 6.10×10^5 , 3.05×10^5 , 1.53×10^5 , 7.63×10^4 , and 3.81×10^4 cells/mL, respectively. Then, the diluted cell suspension was mixed with 50% v/v of two times culture medium, 10% v/v FBS and 40% v/v of CloneMatrix (Molecular Devices, LLC., San Jose, CA, USA, Cat. K8510). For colony formation, the cells in semisolid form were mixed well by vortexing and dispensed into a non-treated 6 well plate at 2 mL/well (Thermo Scientific Nalgene, Waltham, MA, USA, Cat. 150239). The plate was kept at 25 °C in an incubator for 5–9 days to allow single cells to grow into colonies. All colonies were picked from 6 well plates and seeded into 96 well plates using Eppendorf micromanipulator mentioned above. The procedures

are presented in Fig. 2. The 96 well plate containing cloned cells was cultured at 25 °C for 1 month to allow cells to reach confluence. Clones were scaled up using 24 well and finally adapted to culture in flasks.

Biological characteristics of clones

Cell morphology

The cells were observed regularly using an Olympus IX71 inverted microscope with DP72 digital camera. The cell sizes of each cell type were measured by using Olympus CellSens Standard imaging software (version 1.5).

Karyotype analysis

Chromosome spreads of cells were prepared according to the karyotype analysis protocol (Mitsuhashi 2002). The concentration of the hypotonic KCl (Feng Chuan chemical reagent technologies company, Tianjing, China, $\geq 99.5\%$ AT) solution used was 0.6% for the Papilio cell line. The chromosome number of each

Fig. 2 Schematic presenting the semisolid cloning protocol. RIRI-PaDe-3 cells were cultured in semisolid medium, and single cells proliferated in clonal plaques that were then transferred into a 96-well plate using a micromanipulation system

clonal population was determined by counting 100 well-dispersed metaphase spreads. Single-factor analysis of variance (ANOVA) and multiple comparisons were conducted for the chromosome number of cells. The data are presented in histograms.

Inter-simple sequence repeat (ISSR) fingerprint profile analysis

ISSR PCR primers were adapted from Grasela and McIntosh (2003). Four 5'-anchored repeat primers that were used were: ISSR1 (GCT AGT GCT CAC ACA CAC ACA CAT), ISSR2 (GAT GCT GAT GCA CAC ACA CAC ACA), ISSR3 (GCA CAT GCA GTG TGT GTG TGT GTG), and ISSR4 (CAT GCA CAT TGT GTG TGT GTG TGT), and 12 primers containing 3'-terminal repeats: ISSR5 ((GA)₈A), ISSR6 ((AC)₈G), ISSR7 ((GT)₈A), ISSR8 ((AG)₈G), ISSR9 ((AC)₈C), ISSR10 ((AG)₈C), ISSR11 ((CT)₈AC), ISSR12 ((CT)₈GC), ISSR13 ((CA)₆AC), ISSR14 ((CA)₆AG), ISSR15 ((CA)₆GG), and ISSR16 ((CT)₈TG). Each 25 µL PCR reaction used 200 ng extracted DNA from cells, 4 µM primers, and 12.5 µL GoTaq Green Master Mix (Promega Corporation, Madison, WI, USA, Cat. M7122). The PCR conditions were: 95 °C for 3 min; 30 cycles of: 95 °C for 20 s, 40 °C for 20 s, 72 °C for 30 s; and 72 °C for a 5 min final extension. 5 µL of each PCR product was loaded onto a high definition 2% agarose (Biowest, Riverside, MO, USA, Cat. 111760) gel using Bio-Rad PowerPac basic power supply (Hercules, CA, USA, Model 164-5050), run at 40 V for 4 h. For each primer, bands were scored as 1 (presence) or 0 (absence). POPGEN32 (version 1.32) software was used to analyze the genetic diversity of cloned cells, and NTSYSpc (version 2.10e) software was used to determine the genetic distance among cell clones.

Results

Cloning

In this study, 3 cell cloning technologies: limiting dilution, feeder layer culture, and modified semisolid cloning protocol, were used to get monoclonal cultures from RIRI-PaDe-3. The results showed that there was a large difference in the performance of these cell isolation technologies.

Limiting dilution cloning

Ten plates (960 wells) filled with diluted cell suspension were obtained. Only 18 wells that contained just a single cell were detected by microscope. No cell proliferation in the 18 wells was detectable within 8 weeks of culture. During the culture period, crystal precipitations of culture medium were visible at the bottom of the well, and the cells collapsed in some wells. Ultimately, no clonal population was obtained.

Cloning with a feeder layer

Four plates (96 wells) filled with isolated cells were obtained using micromanipulator system. 54 wells with a single cell were identified by microscopy after 4 days. However, black flocs were seen in the wells 2 weeks after providing the feeder layer, and the cells degenerated and died after 33 days of culture. Eventually, no cell clones were obtained using this method.

Cloning with modified semisolid medium protocol

The results showed that the cell-seeding density lower than 3.81×10^4 cells/mL failed to form cell colonies due to an excessively low proliferation of cells, and the cell-seeding density of 1.22×10^6 cells/mL failed to distinguish the edge of each colony because the adjacent colonies were too close and easily adhered to each other. The optimal seeding density of RIRI-PaDe-3 cells was in the range of 3.05×10^5 – 6.10×10^5 cells/mL. The isolation of 480 colonies was achieved using the micromanipulator system. Importantly, a 40% survival rate (192/480 colonies), in 96 well plates was obtained. For the isolated clones, during scale up into 24 well, 5% of 192 clones were lost. Before transferring the cells from 24 well plates to flasks, 40 colonies which were uniform in cell shape were picked up for the next culture, and others were discarded for significant appearance that the cells did not originate from a single cell based on colony form. Eventually, all 40 clones were successfully adapted to culture in flasks.

Comparing three methods, the use of micromanipulator system to pick up single cells or colonies was proven more effective than limiting dilution. Theoretically, the methods of feeder layer and semisolid media could promote the exchange of autocrine growth factors between the single cells prompting

formation of colonies. However, there was only semisolid medium that supported and led to formation of colonies in the current study. These results indicated that the modified semisolid medium cloning protocol was competent to isolate and establish more monoclonal subculture of the Papilio cell line RIRI-PaDe-3.

Biological characteristics of clones

In order to determine whether cloning can lead to cell lines possessing characteristics different from the parent population, 9 cell clones in 40 monoclonal subcultures were evaluated in biological characteristics, morphology, karyotype and genetics background, for their remarkable difference in morphology.

Cell morphology

The RIRI-PaDe-3 cells were mostly attached to the flask and were mainly round, spindle-like (or fibroblast-like), and polygonal (or epithelial-like) as shown in Fig. 3a (Ding et al. 2015). Cells with different morphologies in each cell clone were grouped and counted, and the results are displayed in Table 1. Seven cell clones consisted of mainly round cells, of which RIRI-PaDe-3-C31 (Fig. 3i) had the largest proportion (83.7%). Fibroblast-like cells were dominant in RIRI-PaDe-3-C4 (Fig. 3b), and polygonal and round cells each accounted for half of RIRI-PaDe-3-C27 clones (Fig. 3d). This suggested that there were certain degrees of differences in cell size and cell constitution among cell clones.

Karyotype analysis

The chromosome number of RIRI-PaDe-3 distribution varied widely from 45 to 97 with mean and standard deviation of 68.08 ± 22.078 (Ding et al. 2015). Significant variations in chromosome number were found among the clones (Fig. 4). RIRI-PaDe-3-C4 cells had the largest number of chromosomes (60.03 ± 22.174), and RIRI-PaDe-3-C26 cells (31.60 ± 9.300) had the smallest among the nine cell clones, with the former being approximately double the latter (Table 1). The three cell clones RIRI-PaDe-3-C15, RIRI-PaDe-3-C28, and RIRI-PaDe-3-C41 were similar in chromosome number ($P > 0.05$) but

differed remarkably in cell morphology and constitution.

ISSR fingerprint profile analysis

Sixteen ISSR primers were used for DNA amplification of the nine cell clones, and the amplification products were analyzed by agarose gel electrophoresis. 11 ISSR primers derived satisfactory polymorphic bands and were used to detect the genetic diversity of the nine cell clones (Table 2). The statistical analysis indicated a total of 93 clear bands including 66 polymorphic bands, reaching a polymorphism rate of 70.9%. Primers ISSR5 and ISSR13 achieved a polymorphism rate of 100%. The average genetic diversity index and Shannon's information index of ISSR primers were 0.2315 and 0.3549, respectively. The genetic distance among cell clones was analyzed based on ISSR labeling, and the results are presented in Table 3. The statistical data indicated that there were remarkable genetic differences among cell clones. The average genetic distance was the largest between RIRI-PaDe-3-C8 and RIRI-PaDe-3-C15 (0.4215), indicating the most distant genetic relationship between the two. The distance was the smallest between RIRI-PaDe-3-C27 and RIRI-PaDe-3-C28 (0.0651), indicating the closest genetic relationship between the two.

These results together indicated that the present subcloning of RIRI-PaDe-3 was competent to isolate and establish more authentic cell properties of the parent Papilio cell line, and hence the modified semisolid medium cloning protocol was advantageous to the convenient and genuine cloning from the prior heterogeneous population.

Discussion

The semisolid culture method creates a low-density growing condition where the unique physical property of the semisolid medium minimizes the movement of cells without affecting the exchange of metabolites and communication among cells, thereby facilitating cellular proliferation to form cell colonies (Browne and Al-Rubeai 2007). As the starting number of cells in each well coming from the semisolid medium was high, the time required for cells to grow to confluence in the 96 well plate was shorter. In addition, because

Fig. 3 Photomicrographs of RIRI-PaDe-3 and cell clones (Scale bar: 50 μ m). **a** RIRI-PaDe-3; **b** RIRI-PaDe-3-C4; **c** RIRI-PaDe-3-C8; **d** RIRI-PaDe-3-C15; **e** RIRI-PaDe-3-C26;

f RIRI-PaDe-3-C27; **g** RIRI-PaDe-3-C28; **h** RIRI-PaDe-3-C30; **i** RIRI-PaDe-3-C31; **j** RIRI-PaDe-3-C41

appropriate size of colonies of RIRI-PaDe-3 was too small to be observed and picked up with the unaided eye, picking system such as micromanipulation technique which is usually used in isolating single cells, was performed to transfer colonies. In the present study, we designed a new cloning protocol which integrated the advantages of semisolid culture with efficiency of micromanipulation, and have

successfully applied this protocol in the single cell derived subcloning from RIRI-PaDe-3 cell line. The different sublineage phenotypes between the independent subclones indicate that a facile monoclonal subcloning of genuine clonality is readily attainable by using this method.

Although the limiting dilution method often serves as a simple tool for single-cell cloning, it derived only

Table 1 Morphological characteristics and chromosome numbers of cell clones derived from the cell line RIRI-PaDe-3

Cell name	Chromosome numbers	Attached cell morphology and ratio (%)	
RIRI-PaDe-3-C31	52.83 ± 16.532	Round	83.7
		Spindle-shaped	16.3
RIRI-PaDe-3-C8	40.50 ± 15.024	Round	78.8
		Spindle-like	21.2
RIRI-PaDe-3-C28	36.52 ± 11.843	Round	70.8
		Epithelia-like	29.2
RIRI-PaDe-3-C41	36.07 ± 14.208	Round	70.0
		Spindle-like	21.9
		Epithelia-like	8.1
RIRI-PaDe-3-C30	45.38 ± 16.247	Round	62.6
		Spindle-like	25.1
		Epithelia-shaped	12.3
RIRI-PaDe-3-C15	36.61 ± 13.234	Round	50.3
		Spindle-shaped	49.7
RIRI-PaDe-3-C26	31.60 ± 9.300	Round	49.2
		Short spindle-like	37.5
		Epithelial-like	13.3
RIRI-PaDe-3-C27	56.56 ± 23.694	Polygonal	52.7
		Round	47.3
RIRI-PaDe-3-C4	60.03 ± 22.174	Fibroblast-like	62.0
		Round	38.0

Fig. 4 Number of chromosomes in cells clonally derived from the RIRI-PaDe-3 cell line

single cells that lacked abilities to divide and proliferate and even degenerated and died during culture in this study. Relevant research has found that cells can release growth factors, which promote DNA and protein syntheses and accelerate the mitotic process in cells (Calles et al. 2006a). In an environment with low concentrations of growth factors, it is difficult for

single cells to divide and proliferate. Hence, a certain amount of conditioned medium is usually added into the culture medium to improve the environmental conditions and promote cellular proliferation (Calles et al. 2006b). A feeder layer method was used to mimic a high-density cell growth condition by providing a nurse cell layer, which could increase the communication and interchange of material among cells and enhance division and proliferation of isolated single cells. However, both methods failed to derive viable single-cell clones of RIRI-PaDe-3. A possible cause might be that single RIRI-PaDe-3 cells to be used for clonal culture are too sensitive to the disturbance caused by the isolation operation and thus lack the ability to adapt to the new culture environment for growing.

There were still remarkable morphological differences among cell clones originating from a single cell. The differences might be explained by the previous finding that due to non-synchronous growth, the attached cells are at different stages of mitosis and show certain morphological differences (Grace 1968). In addition, cell phenotypic change is intrinsic during cell culture, and a single cell might differentiate and

Table 2 Parameters for genetic diversity of cloned cells

Primer	Numbers of amplified bands (numbers of polymorphic bands)	Polymorphism rate (%)	Nei's genetic diversity index (H)	Shannon's index (I)
ISSR1	8 (5)	62.5	0.1790	0.2858
ISSR2	9 (2)	22.2	0.0439	0.0775
ISSR3	9 (6)	66.7	0.2431	0.3653
ISSR4	9 (6)	66.7	0.1920	0.3021
ISSR5	6 (6)	100	0.3621	0.5396
ISSR6	7 (4)	57.1	0.1905	0.2887
ISSR8	12 (11)	91.7	0.3086	0.4713
ISSR11	10 (8)	80	0.2617	0.4010
ISSR13	6 (5)	83.3	0.2469	0.3866
ISSR14	4 (4)	100	0.2593	0.4208
ISSR15	13 (9)	69.2	0.2773	0.4055
Total	93 (66)	70.9		
Mean value			0.2315	0.3549

Table 3 Genetic distances among the nine cell clones

Cell clone	RIRI-PaDe-3-C4	RIRI-PaDe-3-C8	RIRI-PaDe-3-C15	RIRI-PaDe-3-C26	RIRI-PaDe-3-C27	RIRI-PaDe-3-C28	RIRI-PaDe-3-C30	RIRI-PaDe-3-C31
RIRI-PaDe-3-C8	0.2189							
RIRI-PaDe-3-C15	0.2517	0.4215						
RIRI-PaDe-3-C26	0.1152	0.1991	0.2319					
RIRI-PaDe-3-C27	0.1239	0.2106	0.2434	0.0699				
RIRI-PaDe-3-C28	0.0945	0.2287	0.2083	0.1101	0.0637			
RIRI-PaDe-3-C30	0.1562	0.2788	0.1982	0.1213	0.1008	0.1008		
RIRI-PaDe-3-C31	0.1289	0.2404	0.2732	0.1268	0.0651	0.0651	0.1044	
RIRI-PaDe-3-C41	0.1998	0.3163	0.3491	0.2348	0.2470	0.2106	0.2788	0.2594

proliferate to form an impure cell population. Therefore, we believe that “cell cloning” is merely an ideal concept and cannot be achieved (Barnes et al. 2001, 2006). ISSR labeling can identify microsatellite differences in genomes to distinguish samples, and potential polymorphisms in genomes can be

determined using different primers (Grasela and McIntosh 2003). In this study, the genetic distance analysis revealed various degrees of genetic differences among the cell clones, theoretically proving that the proposed approach can separate cells with different genetic backgrounds. In fact, genetic information

changes in a portion of cells during long-term culture which can increase the genetic diversity of cell clones (Kasai et al. 2016), thereby providing abundant materials for screening cells that can efficiently express exogenous proteins.

In the present study, 40 cell clones were isolated from the heterogeneous Papilio cell line RIRI-PaDe-3. In future research, their recombinant protein expression will be compared to determine whether cloning can lead to cell lines possessing expression levels superior to the parent population.

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Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest.

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