In Vitro Cell. Dev. Biol.—Animal 32:85–89, October 1996

1071-2690/96 $05.00+0.00

CELL LINES FROM THE MELOLONTHINE SCARAB *ANTITROGUS PARVULUS*

CAROL A. FERNON, REBECCA J. OSBORNE, AND DAVID J. DALL[1]

CSIRO Division of Entomology, GPO Box 1700, Canberra ACT 2601 Australia

(Received 6 March 1995; accepted 10 July 1995)

SUMMARY

Continuously replicating cell lines have been established from embryonic tissue and circulating hemolymph cells of the melolonthine *Antitrogus parvulus* Britton. Isozyme analyses demonstrated that cell lines from both tissue sources expressed essentially the same isoforms of enzymes as *A. parvulus* larvae and thus confirmed the species of their origin. Karyotype analyses showed that cells from both tissue sources had accumulated changes in chromosome number and morphology during culture. Availability of melolonthine-derived cells should assist *in vitro* studies of the pathogens of this important group of beetles.

Key words: Coleoptera, beetle, karyotype, isozyme

INTRODUCTION

Scarab beetles are a cosmopolitan and abundant group, consisting of 30 000 recognized species in 2500 genera (Cassis et al., 1992). They fill a range of ecological niches, with adults and larvae recorded as feeding on plants, humus, carrion, and dung (Lawrence and Britton, 1991). Activities of the dung-feeding and -burying species are considered highly beneficial to humans (Cullen, 1993), but many of the plant-feeding species are recorded as agricultural pests (Jackson, 1992). The subfamily Melolonthinae contains about one-third of all scarab species (Houston and Weir, 1992); its members are commonly known as "chafers," are largely phytophagous, and, in many agricultural systems, are regarded as pests. Larvae of a number of endemic Australian melolonthines, such as *Dermolepida albohirtum* and 18 species of *Antitrogus*, *Lepidiota* and *Rhopaea* are pests of sugarcane (Allsopp et al., 1993).

There have been many attempts to develop biological control agents for soil-dwelling scarab pests (*see* Jackson and Glare, 1992). Investigations of the potential utilities of fastidious intracellular pathogens (e.g., viruses and protozoa) have, however, been hampered by the paucity of scarab cell lines in culture. To date, the only available scarab line is one derived from the dynastid species *Heteronychus arator* (African black beetle) by Crawford (1982). In an effort to produce melolonthine-derived lines for use in propagation of beetle-infecting microorganisms, and specifically as part of a program to develop engineered entomopoxviruses as microbial control agents, we have attempted culture of cells from various melolonthine species. We report here the establishment of continuously replicating lines from hemolymph-derived cells and embryonic tissues of the Childers canegrub, *Antitrogus parvulus* Britton.

MATERIALS AND METHODS

Sources. Adult *Antitrogus parvulus* and larvae of *A. parvulus*, *A. consanguineus*, and *Lepidiota noxia* larvae were collected and identified by Dr. P. G. Allsopp (Bureau of Sugar Experiment Stations, Bundaberg). Larvae of *Dermolepida albohirtum* were collected and identified by Drs. L. N. Robertson and K. J. Chandler (BSES, Tully and Meringa). All specimens were transported by air to the laboratory. Adult *A. parvulus* females laid eggs readily in a substrate of peat moss mixed with potting soil; eggs were recovered and held at room temperature in humidified petri dishes until use. Larvae were held individually in peat moss and potting soil substrate in 150-ml plastic containers and fed carrot.

Media. Cells and tissue explants were maintained in a modified Schneider's medium (Sigma). The basal medium was supplemented with gentamicin (50 µg/ml) and the trace mineral and vitamin mixtures described by Munderloh and Kurtti (1989), and is referred to as Schneider's B medium (SBM).

Lepidopteran *Helicoverpa zea* [BCIRL-Hz-AM1 (McIntosh and Ignoffo, 1981) derived clonal line 2E10; Fernon et al., in press] and *Spodoptera frugiperda* (Sf9; ATCC CRL 1711) cultures were maintained in stationary T-flasks at 27° C in, respectively, TC199MK medium (McIntosh et al., 1973) or Grace's medium (Grace, 1962; Hazelton Research Products, Lenexa, KS) containing 0.25% lactalbumin hydrolysate. Both media were supplemented with 10% fetal calf serum.

Tissue isolation. For each experiment, 7–10 eggs were surface-sterilized by sequential 1–2-min washes in sterile Ringer's solution, 70% ethanol, 1% bleach, then one change of sterile distilled water. After dissection from surrounding shell, embryonic tissue was washed in several changes of SBM containing 5% fetal calf serum (SBM + FCS). The tissue was finely minced in about 150 µl SBM + FCS and spread across the surface of a 25-cm² tissue culture flask (Corning, Corning, NY), which was then turned vertically, facilitating attachment of tissue pieces in a minimum volume of medium. After a brief attachment period (about 1 min) and before pieces became desiccated, 1.5 ml of SBM + FCS was added without disturbing the specimens, the flask was slowly returned to a horizontal position, and the cultures were incubated at 27° C.

Third instar larvae were washed thoroughly with water and then with 70% ethanol before being anesthetized at 4° C. Larvae were then washed again in ethanol and sterile water before a proleg was removed. Approximately 100 µl of hemolymph and circulating cells were collected from each larva into a sterile eppendorf tube, and then transferred to a 25-cm² flask. Cells were allowed to attach for 1–2 min before an equivalent volume of SBM + FCS was added. After overnight incubation (at 27° C), 5 ml of fresh medium was added to each flask.

Replicated attempts were made to establish circulating hemolymph cells of *A. parvulus*, *A. consanguineus* and *L. noxia* in culture. For each species, three separate attempts were made, each with 6–11 larvae which were processed individually (i.e., one larva/flask culture). A total of 86 flask cultures

[1]To whom correspondence should be addressed.

were started and maintained (described later) for 12 weeks or until death of the culture.

Maintenance of cultures. Primary coleopteran explants and outgrowing cells remained in their original flasks until dividing cells were semiconfluent; medium in each flask was replaced at 7-d intervals throughout the incubation period. When possible, we harvested dividing cells for transfer by washing them from the substrate by gentle pipetting or otherwise by a 1-min exposure to 0.5% trypsin (Worthington: Freehold, NJ; Sigma: St. Louis, MO) in calcium- and magnesium-free Hanks' EDTA buffer. New flasks were seeded with 50% of the cells from the original flask. Cultures derived from *A. parvulus* embryonic tissues and hemolymph cells were designated as APE and APHL cells, respectively.

Karyotyping. Preparation of cells for observation of chromosomes essentially followed the method described by Crawford et al. (1983). Briefly, colchicine (0.1 μg/ml, final concentration) was added to flasks containing cultures at 80–90% confluence, and cultures were incubated at 27° C for 5 h. Cells were harvested by mechanical dislodgement without use of trypsin and after centrifugation at 117 × g for 5 min, were resuspended in 0.075 *M* KCl and incubated at room temperature for 30–60 min. Cells were then washed three times with cold Carnoy's solution (methanol: glacial acetic acid, 3:1), placed on a microscope slide, and allowed to air-dry. Cells were stained with 7% Giemsa, and chromosome numbers counted.

Isozyme analysis. Cells from *H. zea* 2E10, *S. frugiperda* Sf9 and *A. parvulus* APE1 and APHL1 lines were harvested, resuspended in water, and stored as aliquots at −70° C. We dissected third instar *D. albohirtum* and *A. parvulus* larvae to remove gut tissue, and the remainder of each cadaver was homogenized in 0.5 ml of cold water. Homogenates from three larvae were pooled, and after centrifugation to remove larval debris, supernatant was stored frozen as described above.

Six enzymes were analyzed: phosphoglucose isomerase (PGI, EC# 5.3.1.9), glucose-6-dehydrogenase (G6PDH, EC# 1.1.1.49), malate dehydrogenase (MDH, EC# 1.1.1.37), malic enzyme (ME, EC# 1.1.1.40), lactate dehydrogenase (LDH, EC# 1.1.1.27), and isocitrate dehydrogenase (IDH, EC# 1.1.1.42). Aliquots of samples from cells and larvae were electrophoresed on horizontal cellulose acetate gels with buffer systems and staining procedures described by Herbert and Beaton (1989), with some modifications. Briefly, G6PDH analysis was done with acetate gels presoaked for 15 min in 150 ml of Tris-glycine (pH 8.5) running buffer containing 15 mg nicotinamide adenine dinucleotide phosphate and 0.5 ml of 1 *M* $MgCl_2$ and gels for MDH analysis were presoaked for 15 min in 150 ml of running buffer containing 15 mg nicotinamide adenine dinucleotide (Richardson et al., 1986).

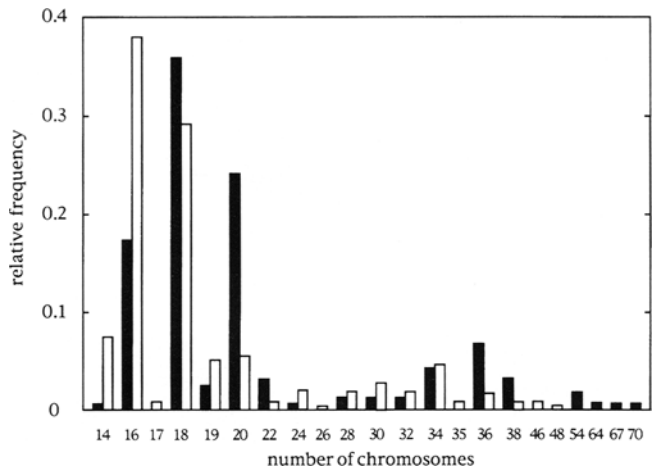

FIG. 2. Frequency distribution of chromosome numbers in metaphase spreads of *Antitrogus parvulus* APHL1 (*shaded*; n = 161) and APE1 (*hollow*; n = 253) cells in culture.

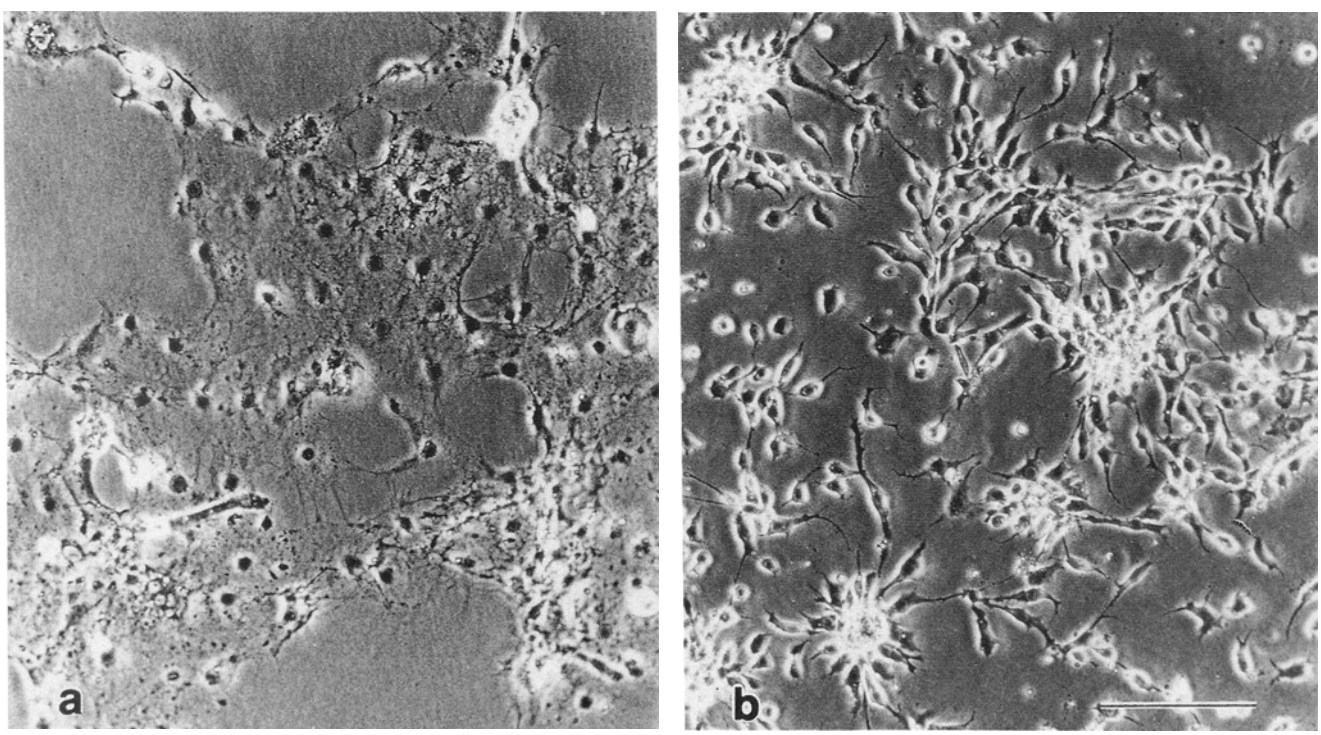

FIG. 1. (*a*) *Antitrogus parvulus* APHL1 cells; *note* highly flattened morphology and sheet-like configuration. (*b*) *A. parvulus* APE1 cells; *note* predominant elongate morphology and clumped configuration. *Scale bar* shows 200 μm.

TABLE 1

ISOZYME PROFILES OF INSECT CELL LINES AND TISSUES

Source	Isozyme[a]					
	PGI	MDH	G6PDH	ME	LDH	IDH
Antitrogus parvulus						
APE1 line	157	100	−375, −475	73, 111	108	25, 56
APHL1 line	142	100	−525	73, 111	108	25, 56
Larval tissue	157	100	−525	111	108	25, 56
Dermolepida albohirtum						
Larval tissue	43	125	−475	111	166	25
Helicoverpa zea						
line Hz-AM1, clone 2E10	43	100, −500	25	94	141	93
Spodoptera frugiperda[b]						
line Sf9	100	100, −500	100	100	100	100

[a]PGI, phosphoglucose isomerase; MDH, malate dehydrogenase; G6PDH, glucose-6-dehydrogenase; ME, malic enzyme; LDH, lactate dehydrogenase; IDH, isocitrate dehydrogenase.

[b]Isozymes of Sf9 cells were used as internal standards and assigned a relative mobility of 100. Sf9-derived G6PDH migrated cathodally.

Isozymes of Sf9 cells served as internal standards in all assays. The predominant band of each isozyme in the Sf9 line was assigned a mobility value of 100, and the direction and extent of movement of isozyme bands in all other lines was then expressed relative to this standard (Tabachnick and Knudson, 1980).

RESULTS

Establishment of Hemolymph-Derived Cells

Observation by light microscopy showed that the majority of cells collected in larval hemolymph were plasmatocytes and granulocytes (Jones, 1977). Although proportions of these two cell types varied between individual larvae, granulocytes generally predominated. Granulocytes did not, however, attach to the flasks; in general they persisted in cultures for 1–2 weeks, during which time the majority of granule contents were apparently released into the medium. Most deteriorating granulocytes were thus removed from new cultures during the first few changes of medium. In contrast, plasmatocytes attached firmly to the substrate within 24 h of collection and survived in culture for considerably longer periods.

Cultures of *A. consanguineus* hemocytes survived relatively poorly under conditions used in this study and never persisted for more than 7 weeks. In contrast, hemocytes from *L. noxia* survived extremely well, and more than 50% of cultures were alive 12 weeks after being started. Nevertheless, none showed signs of continuous division, and maintenance of these cultures was ultimately discontinued. Hemocytes from *A. parvulus* also survived well, and actively growing cells were subcultured as early as 3 weeks after cultures began. Although most of those did not survive indefinitely, cells from one culture continued to replicate, giving rise to line APHL1. The APHL1 line has now survived for more than 2 years in continuous culture, during which time it has been passaged 25 times. Repeated subculturing of replicating cells from the progenitor culture gave rise to a series of APHL1 sublines which are being further characterized.

Establishment of Embryonic Cells

Attempts to culture cells derived from *Antitrogus parvulus* embryos before yolk enclosure proved more successful than those with older embryos. Cells from the former source showed very different responses in the two media formulations tested [L15B with 1% FCS (Munderloh and Kurtti, 1989), and SBM + FCS]; in L15B + 1% FCS, survival times exceeded 12 months, but without significant levels of cell increase, whereas in SBM + FCS, outgrowth from tissue explants was evident after as little as 4 weeks of incubation.

One embryo-derived culture showed a high level of cell multiplication and was the progenitor of line APE1. The APE1 line has survived more than 2 years in culture and has been passaged more than 20 times. We have generated a "loosely attached" line (APE1L) from line APE1 by passaging cells dislodged from the substrate by gentle washing with a pipette, and this and five other sublines from that progenitor culture are now being studied further.

Characterization of Lines APHL1 and APE1

Morphology. APHL1 cells (Fig. 1 *a*) typically showed a very highly flattened phenotype and commonly assumed a multicellular sheet-

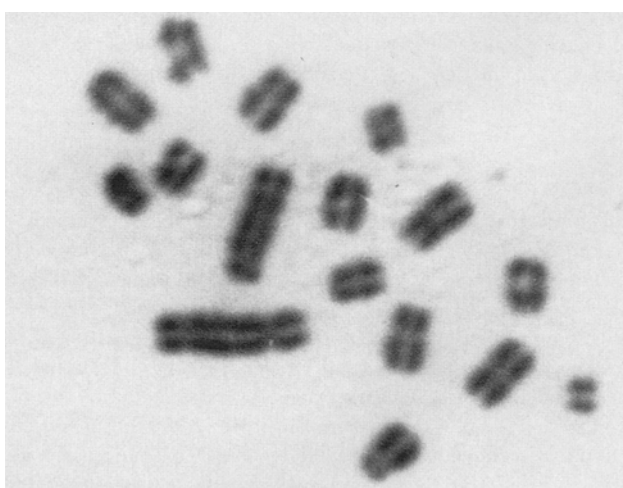

FIG. 3. Representative karyotype of metaphase chromosomes of *Antitrogus parvulus* APE1 cells.

like configuration across the substrate. Floating spindle-shaped APHL1 cells occurred in the medium when cultures were allowed to approach confluence, but these attached and assumed the typical flattened morphology after subculturing. The flattened morphotype appears to be stable, having to date been maintained through more than 15 passages. The cells are firmly attached to the culture vessel and generally require trypsin treatment for successful harvesting. Embryo-derived APE1 cells initially showed three common morphotypes (spindle-shaped, elongated, and elongate with branched processes), but with continued passaging their morphology became more uniform, and eventually cultures were produced composed largely of monolayers of firmly attached spindle-shaped cells. About 12 months after establishment, further morphological changes resulted in production of cultures composed of loosely attached cells with a tendency to clump (Fig. 1 *b*). This morphology was also displayed by the deliberately selected, loosely-attached cell line APE1L. It is apparently stable and has been retained over 12 months (15 passages) to the present.

Isozyme analysis. We assessed the isozyme profiles of a number of metabolic enzymes in extracts prepared from *A. parvulus* and other insect cells. Six of those enzymes could be reliably detected in each of the extracts tested, and their profiles are shown in Table 1. In addition, we made a detailed analysis of lactate dehydrogenase (LDH) isozymes in extracts from a number of APE1 and APHL1 subcultures which had been isolated at different times from the progenitor cultures and separately maintained. Preparations from those cells contained a single isozyme band of consistent mobility (Table 1), irrespective of their tissue of origin (i.e., embryonic or hemocyte), point of temporal derivation from progenitor culture, or passage number. Isozymes in samples from cultured *A. parvulus* cells corresponded well to those from larval *A. parvulus* tissue at both the LDH and some other (MDH, IDH) loci and thus allowed discrimination between materials of *A. parvulus* and other origin.

Karyotype analysis. Chromosome numbers in APHL1 and APE1 cells were highly variable. Counts on 414 cells (Fig. 2) showed that cells with 16 or 18 chromosomes predominated in the cultures, with the former being the modal number for cells of the APE1 line and the latter for APHL1 cells. A smaller but distinct cluster of cells with 30–38 chromosomes was also observed, and a maximum number of 70 was recorded. Although chromosome morphology appeared unremarkable in a majority of cells (Fig. 3), abnormalities such as dicentric and ring forms were observed in some spreads.

Discussion

Work reported here extends the availability of coleopteran cells in culture to the large and economically important melolonthine subfamily of scarabs. The major taxa used in this study (*Antitrogus parvulus, A. consanguineus, Lepidiota noxia*) are all members of the tribe Melolonthini and are thus considered closely related; even so, our studies revealed clear differences in, first, the capacities of their plasmatocytes to persist *in vitro* under the conditions tested, and secondly, their isozyme marker profiles.

One aim of our work was to investigate the possibility of describing a culture medium widely suitable for use in melolonthine studies. However, based on the varied response of plasmatocytes from different taxa to maintenance and growth in Schneider's B medium, it seems unlikely that this will be feasible. Rather, an empirical approach to optimizing media composition for cells from different melolonthine sources is likely to prove necessary. In this respect, melolonthines would then resemble other more intensively studied insect taxa (e.g., the noctuid lepidopterans), whose cells in culture display media preferences in an essentially unpredictable manner.

Characterization of isozyme profiles of six enzymes from various *in vivo* and *in vitro* sources gave results consistent with the derivation of APE1 and APHL1 cells from an *A. parvulus* origin. Isozyme profiles of the enzymes MDH, LDH, and IDH allowed differentiation between all *A. parvulus*-derived materials and those from another melolonthine, *Dermolepida albohirtum*, and profiles of all six enzymes we tested separated the *A. parvulus* lines from others of lepidopteran origin routinely used in our laboratory.

Analyses of karyotypes of cells from *A. parvulus* lines showed an accumulation of variation in both chromosome number and morphology, a phenomenon common to many insect cell lines and also documented previously for the scarab-derived line DSIR-HA-1179 (Crawford et al., 1983). Although there is no description available of the chromosome complement of *A. parvulus in vivo*, studies of other melolonthines have documented haploid numbers of 9–11, with a modal number of 10 (Yadav and Pillai, 1979). On that basis, a majority of cells in our lines are probably aneuploid with a smaller percentage representing some state close to tetraploidy.

A. parvulus is only the second scarab species from which cells have been cultured. Availability of the lines described here should facilitate *in vitro* studies of a range of arthropod pathogens, and in particular, of agents which might eventually be useful in the biological control of scarab pests.

Acknowledgments

We thank Drs. P. G. Allsopp, L. N. Robertson, and K. J. Chandler (Bureau of Sugar Experiment Stations) for material and advice, Mrs. T. M. Symonds for technical assistance, and the Sugar Research and Development Corporation for financial support.

References

Allsopp, P. G.; Chandler, K. J.; Samson, P. R., et al. Pests of Australian sugarcane. Bureau of Sugar Experiment Stations; 1993:90.

Cassis, G.; Houston, W. W. K.; Weir, T. A., et al. Scarabaeoidae. In: Houston, W. W. K., ed. Zoological catalogue of Australia. Coleoptera: Scarabaeidae. Canberra: Australian Government Publishing Service; 1992:1–3.

Crawford, A. M. A coleopteran cell line derived from *Heteronychus arator* (Coleoptera: Scarabaeoidea). In Vitro 18:813–816; 1982.

Crawford, A. M.; Parslow, M.; Sheehan, C. Changes in the karyotype of the cell line DSIR-HA-1179, and a comparison with that of its parent insect, *Heteronychus arator* (F.) (Coleoptera: Scarabaeoidea). N. Z. J. Zool. 10:405–408; 1983.

Cullen, J. M. Opportunities and challenges in biological control. In: Corey, S. A.; Dall, D. J.; Milne, W. M., eds. Pest control and sustainable agriculture. Melbourne: CSIRO Australia; 1993:44–50.

Fernon, C. A.; Vera, A. P.; Crnov, R., et al. Replication of *Heliothis armigera* entomopoxvirus *in vitro*. J. Invertebr. Pathol.; in press.

Grace, T. D. C. Establishment of four strains of cells from insect tissues grown *in vitro*. Nature 195:788–789; 1962.

Herbert, P. D. N.; Beaton, M. J. Methodologies for allozyme analysis using cellulose acetate electrophoresis. Helena Laboratories; 1989:32.

Houston, W. W. K.; Weir, T. A. Melolonthinae. In: Houston, W. W. K., ed. Zoological catalogue of Australia. Coleoptera: Scarabaeoidea. Canberra: Australian Government Publishing Service; 1992:174–182.

Jackson, T. A. Scarabs—pests of the past or the future? In: Jackson, T. A.; Glare, T. R., eds. Use of pathogens in scarab pest management. Hampshire: Intercept; 1992:1–10.

Jackson, T. A.; Glare, T. R., eds. Use of pathogens in scarab pest management. Hampshire: Intercept; 1992:209.

Jones, J. C. The circulatory system of insects. Illinois: Charles C. Thomas Publishers; 1977:116.

Lawrence, J. F.; Britton, E. B. Coleoptera. In: Naumann, I.D., ed. The insects of Australia. Second edition. Melbourne: Melbourne University Press; 1991:543–683.

McIntosh, A. H.; Ignoffo, C. M. Replication and infectivity of the single-embedded nuclear polyhedrosis virus, *Baculovirus heliothis*, in homologous cell lines. J. Invertebr. Pathol. 37:258–264; 1981.

McIntosh, A. H.; Maramorosch, K.; Rechtoris, C. Adaptation of an insect cell line (*Agallia constricta*) in a mammalian cell culture medium. In Vitro 8:375–378; 1973.

Munderloh, U. G.; Kurtti, T. J. Formulation of medium for tick cell culture. Exp. App. Acar. 7:219–229; 1989.

Richardson, B. J.; Baverstock, P. R.; Adams, M. Allozyme electrophoresis. Sydney: Academic Press; 1986:410.

Tabachnick, W. J.; Knudson, D. L. Characterization of invertebrate cell lines. II. Isozyme analyses employing starch gel electrophoresis. In Vitro 16:392–398; 1980.

Yadav, J. S.; Pillai, R. K. Evolution of karyotypes and phylogenetic relationships in Scarabaeidae (Coleoptera). Zool. Anz. 202:105–118; 1979.