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ICGEB Research Grants Programme

RESEARCH GRANTS COMPLETED IN 2016

CRP - ICGEB RESEARCH GRANTS COMPLETED IN 2016

COUNTRY	PRINCIPAL INVESTIGATOR	PROJECT TITLE
BRAZIL	Guido LENZ	The role of autophagy in the *in vivo* growth and response to therapy in gliomas
MALAYSIA	Azah Kamilah MUDA	A new 3D descriptor of synthetic drug molecular structure for drug analysis
PERU	Gisella ORJEDA	Races of rust affecting barley production of Andean farmers
SRI LANKA	Aloka Lanka RANAWAKE	Resolving cultivar identities in Sri Lanka traditional rice accessions using SSR markers
URUGUAY	Pablo S. AGUILAR	Yeast mating as a model for cell-cell fusion
VENEZUELA	Mercedes FERNÁNDEZ MESTRE	Study of genetic susceptibility of infectious diseases
VIET NAM	Thanh Hoa LE	Mitogenomic analysis of neglected tropical parasitic heterophyid and paragonimid zoonoses for diagnostic and epidemiological application in Viet Nam

BRAZIL

Title: The role of autophagy in the *in vivo* growth and response to therapy in gliomas
Principal Investigator: Guido Lenz, LabSinal, Department of Biophysics, Universidade Federal do Rio Grande do Sul, Av. Bento Goncalves, Porto Alegre, RS, Brazil. Tel: +55-51-33087613, E-mail: lenz@ufrgs.br
ICGEB Contract No.: CRP/12/004
ICGEB Reference No.: CRP/BRA11-01
Abstract: Autophagy is a process that recycles cellular components through the lysosome, degrading faulty components and producing energetic molecules. Thus, autophagy is activated by several cell stresses that increase the proportion of faulty components and by starvation. Tumour cells submitted to chemotherapy normally induce autophagy to different degrees. The central question of this grant was to investigate the role played by autophagy in the therapeutic action of Temozolomide (TMZ) *in vitro* and *in vivo* in gliomas.
First we established that a short pulse of TMZ, the chemotherapy of choice to treat gliomas, induces a transitory increase of autophagy in glioma cells over a period of 7 days, indicating a chronic response to the acute stress induced by TMZ. Inhibiting autophagy at early stages of the process at days 3, 4 and 5 after TMZ increased apoptosis, indicating a protective role of autophagy in a proportion of cells. Interestingly, inhibiting autophagy at the late stage of the process did not increase apoptosis, indicating different outcomes of blocking autophagy early or late. Inducing autophagy, on the other hand, increased the proportion of glioma cells entering senescence in response to TMZ. Since both senescence and apoptosis restrict tumour growth, we established a protocol in which autophagy is induced prior to TMZ treatment to increase senescence and autophagy is blocked 3, 4, and 5 days after TMZ treatment, to increase apoptosis. This combination of inducing autophagy by pre-treating cell with Rapamycin, treating cells with TMZ followed by the inhibitor of autophagy 3MA led to a very large reduction of cells. This combination was tested in an *in vivo* model of glioma growth and preliminary results indicate that this combination also strongly delays tumour growth when compared to TMZ alone. Silencing key autophagy genes did not alter sensitivity, probably because these reduces autophagy systematically. Therefore, the first contribution of the work supported by the present grant was to show that a fine tuned modulation of autophagy involving both induction and inhibition at different times in relation of the chemotherapeutic agent is important to consider in the use of modulators of autophagy as a chemosensitiser in chemotherapy.
One of the central biological questions regarding the above results is the molecular mechanism of the connection between autophagy and senescence. All studies regarding this connection were based on population of cells and did not clarify the role of autophagy in the induction of senescence. We tracked single TMZ-treated cells from day 3 to 5, which is the period in which autophagy decreases and senescence strongly increases in the whole population. Opposite to data from the whole population, autophagy and senescence did not correlate at a single cell level. Furthermore, the decrease of autophagy was not necessary to senescence induction, and cells decrease autophagy after, before or concomitant to the acquisition of senescent phenotype. The second contribution of this grant was to show that in single cells, autophagy and senescence are not interdependent and that both mechanisms were triggered by TMZ treatment, but occurred with different kinetics.
The grant was fundamental for the development of the Ph.D. thesis of Eduardo C. Filippi-Chiela. It also greatly contributed for the Master degree work of Marcos P. Thomé, the undergrad work of Mardja M.B. Silva, the post-doc of Karina B. Felipe and collaborating Ph.D. student Paola Mello. Additionally, the grant contributed for the maintenance and basic reagents of a large group of 20 students.
The research conducted with the funds provided by the present grant were published in Autophagy entitled "Single-cell analysis challenges the connection between autophagy and senescence induced by DNA damage". Additionally, this grant supported indirectly the paper published in Molecular Biology of the Cell entitled "Uptake of adenosine induced autophagy through phosphorylation by adenosine kinase and activation of AMPK". The experience our group gained in autophagy with the development of this grant certainly contributed for the publishing of a review in Cell Death and Differentiation entitled "Autophagy and Genomic Integrity" and the participation as authors in the "Guidelines for the use and interpretation of assays for monitoring autophagy" recently published in the journal Autophagy.
Objectives:
The central question of this grant was to investigate the role played by autophagy in the therapeutic action of TMZ *in vitro* and *in vivo* in gliomas.
Results Obtained:
Temozolomide (TMZ), induced autophagy in glioma cells and inhibition of this autophagy at early stages after TMZ increased apoptosis, whereas inhibiting autophagy at the late stage, did not

increase apoptosis, indicating different outcomes of blocking autophagy early or late. Inducing autophagy increased the proportion of glioma cells entering senescence in response to TMZ. Combination of autophagy induction prior to TMZ treatment with autophagy block after TMZ treatment led to a very large reduction of glioma cells *in vitro* and a delayed *in vivo* growth when compared to TMZ alone. Silencing key autophagy genes did not alter sensitivity, probably because this reduces autophagy systematically.

To better understand the role of autophagy in the establishment of senescence, we tracked single TMZ-treated cells from day 3 to 5, which is the period in which autophagy decreases and senescence strongly increases in the whole population. We found that the decrease of autophagy was not necessary to senescence induction, and cells decrease autophagy after, before or concomitant to the acquisition of senescent phenotype, establishing autophagy and senescence as independent processes in cells treated with TMZ.

Results Unforeseen in the Original Project:

The importance of activating and inhibiting autophagy to sensitise cells to chemotherapy.

Publications:

Filippi-Chiela, E.C., Vessoni, A.T., Menck, C.F.M., Lenz, G. Autophagy and genomic integrity. 2013. Cell Death Differ. **20**, 1444-1454

Mello, P.D.A., Filippi-Chiela, E.C., Nascimento, J., Beckenkamp, A., Santana, D.B., Kipper, F., Casali, E.A., Nejar Bruno, A., Paccez, J.D., Zerbini, L.F., Wink, M.R., Lenz, G., Buffon, A. Adenosine uptake is the major effector of extracellular ATP toxicity in human cervical cancer cells. 2014. Mol. Biol. Cell. **25**, 2905-2918

Filippi-Chiela, E.C., Bueno e Silva, M.M., Thome, M.P., Lenz, G. Single-cell analysis challenges the connection between autophagy and senescence induced by DNA damage. 2015. Autophagy **11(7)**, 1099-1113

MALAYSIA

Title: A new 3D descriptor of synthetic drug molecular structure for drug analysis
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ICGEB Contract No.: CRP/13/010
ICGEB Reference No.: CRP/MYS13-03
Abstract: Drug abuse is a threat to development. It affects society, economy and nation growth. The abuse of synthetic drugs such as ATS drugs has become harrowing problem. There are still lack concrete solution in identifying unfamiliar ATS drug substances. Soft computing technique can be used as an alternative solution to complement GC/MS lab experiment to identify existing and new substance of ATS drug. Generally, drugs can be identified based on 2D and 3D shape of its molecular structure. However, 3D shape descriptor has richer characteristics of volume and surface in recognising the molecular structure. Hence, this research aims to formulate a new 3D molecular structure descriptor of Legendre Moment to identify unique characteristic of synthetic drug. The proposed 3D Exact Legendre Moment Invariants descriptor is able to represent 3D molecular structure in computational numerical format with higher accuracy compared to other benchmarking Moment Function methods. Accurate 3D representation is crucial in recognising complex shape of molecular structure due to rotation invariance of molecular surface. The outcome of this project will be used to produce an automatic descriptor for ATS drug in future work.
Objectives:
(i) To formulate a new 3D mathematical model of 3D molecular structure descriptor in drugs analysis;
(ii) To validate the proposed 3D molecular structure descriptor in transforming the molecular structure of drug into computational invariant feature representation.
Results Obtained:
The research has been successfully proposed a new molecular descriptor method, namely 3D Exact Legendre Moment Invariants. Although the proposed method is not as good as existing molecular descriptors when Tanimoto coefficient similarity measurement is employed, the promising applicability of the proposed method has been demonstrated and worth to receive further exploration in identifying unknown ATS drugs. On the other hand, the proposed method is almost at par with the existing molecular descriptors when Random Forest classifier is used.
The literature review conducted has shown that there is a Moments Function that has discriminative property on par with 3D Zernike Moments, which is 3D Legendre Moments. Some of these orthogonal moments are only proposed to deal with 2D image, not 3D image. Therefore, prior to determining the fittest orthogonal moments, these orthogonal moments must be enhanced into 3D version. After these orthogonal moments has been enhanced and developed, the comparative study has been conducted. Non-orthogonal moments, such as 3D Geometric and 3D Complex Moments are also employed in the comparison as the benchmarking result. Conclusively, it is found that 3D Legendre Moments performs better compared to other orthogonal moments, in terms of classification accuracy, memory usage, and processing time. And thus, the focus of this research is towards 3D Legendre Moments.
The proposed method is first compared to the existing 3D Geometric Moment Invariants. However, due to the limited number of dataset, it is required to obtain additional ATS and non-ATS drug molecular structures. A total of 7080 molecular structures were obtained, with 3540 molecular structures for both ATS and non-ATS each. After comparison has been conducted using well-known machine learning classifier, it is found that 3D Exact Legendre Moment Invariants performed better than 3D Geometric Moment Invariants (83.70% to 74.11% respectively), and the results was also validated using statistical method, which yield statistically significant difference. Therefore, in this stage, it is concluded that the proposed method is a better shape descriptor compared to the benchmark method.
The next validation it to test whether the proposed method performs better as a molecular descriptor compared to the existing and commonly molecular descriptors, such as Weighted Holistic Invariant Molecular (WHIM) Descriptors and 3D Zernike Descriptors, by benchmarking it using state-of-the-art similarity measurement in the cheminformatics domain. However, the test reveals that the proposed method is not as good as the existing methods (64.94% for the 3D Exact Legendre Moment Invariants, 84.26% for the WHIM Descriptors, and 88.87% for the 3D Zernike Descriptors). Further analysis of the results show that the underperformance of the proposed method is due to the large number of features produced (1185 features), as opposed to relatively smaller number of features of the existing methods (91 and 121 features for WHIM and 3D Zernike Descriptors, respectively). Therefore, another study to select the most discriminative features to improve the accuracy of the proposed method is required. On the other hand, the comparative study conducted using Random Forest classifier showed that the proposed method is at par with

the existing methods (83.70% for the 3D Exact Legendre Moment Invariants, 89.68% for the WHIM Descriptors, and 84.27% for the 3D Zernike Descriptors). It is also concluded that the underperformance of 3D Exact Legendre Moment Invariants is not merely due to the number of features, but also influenced by the similarity measurement employed. Hence, it is also necessary to conduct another study to determine the best similarity measurement to be employed, and possibly produce a novel similarity measurement method to be employed in cheminformatics domain. But nevertheless, the Objective 2 of this study has been successfully achieved.

Results Unforeseen in the Original Project:

The research was originally proposed to extend 2D United Moment Invariants, however it is found that 2D UMI does not explain the procedure to produce the moment invariants, hence it is difficult to generalise it into *n*-dimensional moments. Moreover, 2D UMI does not employ higher order moments, and thus only capable to produce limited number of features. Therefore, the focus of this research is now shifted to find another Moments Function, which leads to the development of the 3D Exact Legendre Moment Invariants. The research also produced a new tool to efficiently convert 2D molecular structure into 3D molecular structure, which was not originally proposed in the original project.

Publications:

Pratama, S.F., Muda, A.K., Choo, Y.H., Abraham, A. A comparative study of 2D UMI and 3D Zernike shape descriptor for ATS drugs identification. 2015. In: Pattern Analysis, Intelligent Security and the Internet of Things, Springer International Publishing, 237-249

Pratama, S.F., Muda, A.K., Choo, Y.H., Abraham, A. Exact computation of 3D geometric moment invariants for ATS drugs identification. 2016. In: Innovations in Bio-Inspired Computing and Applications, Springer International Publishing, 347-358

Saw, Y.C., Muda, A.K. An overview of computational intelligence technique in drug molecular structure identification. 2016. In: Innovations in Bio-Inspired Computing and Applications, Springer International Publishing, 473-480

PERU

Title: Races of rust affecting barley production of Andean farmers

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Abstract: Stripe rust-resistant barley varieties have been evaluated in experimental fields at the USA, Mexico and Peru (Rossi, 2006). Resistant varieties brought from the USA assessed in fields of Peru showed both resistance and susceptibility, depending on the area where they were evaluated. Apparently, the existence of endemic races for *Puccinia striiformis* f. sp. *hordei* (PSH), prevents transferring the results of international research to the communities most vulnerable to economic losses due to this pathogen.

Due to the poor adaptation and asynchronous growth habits of these cultivars, field assessment with differentials would not have been efficient. We used a population genetics approach and profit from the availability of molecular markers to assess the unknown number of races of PSH. We also determined the distribution of these races in the highlands of Peru.

Additionally, this project provided training opportunities at the Principal Investigator's Institution, as well as the collaborating Institution in the highlands of Peru, for young scientists from the institutions involved regarding plant pathology and molecular population genetics of pathogens.

Objectives:

The general objective was to identify molecular and morphological races of *Puccinia striiformis* f. sp. *hordei* (PSH) and assess their virulence and geographical distribution in the highlands of Peru, specifically in Ayacucho (2,746 MASL).

Specific objectives were as follows:

(i) to create a working collection of local samples of PSH;

(ii) to develop protocols to create monosporic cultures of the pathogen and maintain them in the laboratory;

(iii) to standardise molecular identification of the pathogen through PCR;

(iv) to characterise the biodiversity of the pathogen in order identify putative populations that might evade current introduced resistance in barley cultivars;

(v) to train human resources in molecular phytopathology and biodiversity assessment;

(vi) technology transference to Ayacucho in the highlands of Peru.

Results Obtained:

A collection of 150 stripe rust sample is kept at UPCH. We now have a standardised protocol to identify stipe rust populations through DNA sequence. We have identified two sub-populations (i.e. putative races) of the pathogen and have mapped their distribution. Additionally, we have trained 3 graduate students from the Universidad Peruana Cayetano Heredia (Lima) and 2 undergraduate students from the Universidad Nacional de San Cristóbal de Huamanga (Ayacucho). Four of them used the results of this project for their thesis.

Results Unforeseen in the Original Project:

It was impossible to standardise a method to develop monosporic cultures in our labs because it is very hard to cultivate biotrophic fungi.

SRI LANKA

Title: Resolving cultivar identities in Sri Lanka traditional rice accessions using SSR markers
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ICGEB Contract No.: CRP/13/019
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Abstract: Traditional rice cultivars in Sri Lanka have been collected and deposited in Plant Genetic Resources Center (PGRC), Gannoruwa, Peradeniya, Sri Lanka for future utilisation. These rice cultivars have been collected based on their designated names in the area of collected. There are many rice accessions in PGRC collection catalogued in the same name but given different accession numbers. As an example, many rice accessions are available in the PGRC collection by the name of popular salinity tolerant rice cultivar *Pokkali.* However, it was found that all the *Pokkali* rice accessions responded for salinity in different levels. Similar phenomenon was reported in many different rice accessions, designated by the same name: *Dahanala* (6739, 5386, 3917, 3131), *Dik Wee* (3444, 3741, 4927, 3504, 2203), Heenati (3707, 3936, 3998, 4618, 4935, 6402, 4524), *Kalu Wee* (3212, 3728, 3876, 4624), *Kalubala Wee* (5480, 3976, 5479, 5481, 3158, 3172), *Kalu Heenati* (5384, 3851, 3471, 4621, 4991, 7802, 5191), *Kuru Wee* (3465, 3552, 3898, 3982, 4679), *Maa Wee* (3618, 3683, 3704, 8548, 8551, 8552, 8497, 5531, 4145, 5384), *Murungakayan* (3921, 3900, 6263, 6285, 3809, 3495, 3490, 3489, 3492), *Podi Wee* (3539,10109, 3145), *Pokkali* (3881, 3922, 3701, 3573, 3567, 3562), *Polayal* (3639, 3661), *Rata Wee* (3466, 3525, 3655, 4580), *Rathu Wee* (3473, 3905, 4992), *Rathu Heenati* (5486, 4992, 6249), *Sudu Heenati* (4932, 5670, 7799, 3932), and *Suduru Samba* (2202, 4362, 3671).
Morphological diversity of selected traditional rice accessions with the same name were evaluated at the field conditions in *Maha* seasons 2012/2013 and 2013/2014 at the Faculty of Agriculture, University of Ruhuna, Mapalana, Sri Lanka. Ten days old seedlings were transplanted in rows with 15 cm x 20 cm spacing according to the randomised complete block design (RCBD), in 4 triplicates, having 3 rows per replicate and 20 plants per each row. Plant height, number of tillers/plant, number of fertile tillers/plant, panicle length, panicle weight, filled grains/panicle, total grains/panicle, filled grain percentage, 100 grain weight, total grain weight/plant, biomass, and harvest index were recorded according to the Standard Evaluation System (SES) for Rice. "Days to flowering" was counted at 50% flowering. Data were statically analysed using SPSS software.
Commercially available primer pairs for rice SSRs were used for polymorphism survey using extracted DNA from rice cultivars as templates. SSR markers were amplified in a 96-well plate using the Gene Amp PCR System 9700 thermocycler. PCR conditions were as follows: 5 minutes at 95 ^{o}C, 35 cycles of 95 ^{o}C for 1 minute, 55 ^{o}C for 1 minute and 72 ^{o}C for 2 minutes, with a final extension at 72 ^{o}C for 7 minutes. Number of SSR markers with polymorphic bands are being used for further analysis.
According to morphological dendrogram rice accessions were clustered into homogenised groups at different cluster distances. More polymorphic SSR markers must be added to analyse the genetic variation among studied rice accessions designated from the same name.
Data collected from the research and background knowledge were compiled into books. Survey on traditional rice cultivars in remote areas of Sri Lanka was completed in some areas and few more areas will be covered in coming days. Publication of research findings were done as books, journal publications, International and national symposia namely 10th International Conference on Healthcare and Biological Research (ICHBR), Asian Institute of Technology (AIT), Conference Center, Bangkok, Thailand, 3rd Ruhuna International Science and Technology Conference (RISTCON), Faculty of Science, University of Ruhuna, Matara, Sri Lanka, 13th Academic Sessions, University of Ruhuna, Matara, Sri Lanka, International symposium on Agriculture and environment (ISAE), Faculty of Agriculture, University of Ruhuna, Sri Lanka, and 3rd International Research Symposium (IRSyRUSL), Faculty of Management studies, Rajarata University of Sri Lanka.
A postgraduate student was trained in the fields of data collection, data analysis, writing scientific communications: poster presentations and oral presentations. Several papers were published in the field of traditional rice cultivars concluding data of parallel studies. Financial assistance for attending an international conference for both the postgraduate student and the PI was provided by the research grant and PI attended a seminar for a presidential address (Seminar on "Integrating Agriculture & Allied Research: Prioritising Future Potentials for Secure Livelihoods" (ISIAAR) BCKV, Bangalore, India) on "Researches on screening and development of stress tolerant rice cultivars for Food and Nutritional Security under Changing Climatic Conditions", organised by the Crop and Weed Science Society (CWSS), Bidhan Chandra Krishi Viswavidyalaya (BCKV) Bangalore, India.
Objectives:
(i) Collect traditional rice cultivars with the same name in Sri Lanka;

(ii) Seed multiplication and field testing;
(iii) Characterisation of rice cultivars according to field performances;
(iv) Identification of morphological similarities and diversities of traditional rice cultivars with the same name by collecting data on Plant height, tiller number, number of fertile tillers/plant, days to flowering, panicle length, 100- seed weight, seed length, seed weight, yield/plant;
(v) Identification of morphological diversities of rice cultivars with different names;
(vi) Identification of genetic diversity of traditional rice cultivars using SSR markers;
(vii) Compilation of research data in to a book both in English and Sinhala;
(viii) Survey on traditional rice cultivars in remote areas of Sri Lanka;
(ix) Publication of research finding;
(x) Training for a post graduate student;
(xi) Financial assistance for page charges;
(xii) Financial assistance for attending international conferences;
(xiii) Training on scientific writing

Results Obtained:

(i) Collect traditional rice cultivars with the same name in Sri Lanka: Sixty five traditional rice accessions in 14 different names were able to select from the collection available at plant genetic resources centre, Gannoruwa, Peradeniya, Sri Lanka.
(ii) Seed multiplication and field testing: The field experiments were carried out in Maha seasons 2012/2013 and 2013/2014 at Faculty of Agriculture, University of Ruhuna, Mapalana, Sri Lanka.
(iii) Characterisation of rice cultivars according to field performances: Ten days old seedlings were transplanted in rows with 15 cm x 20 cm spacing according to the randomised complete block design (RCBD), in triplicates, having 3 rows per replicate and 20 plants per each row. Plant height, number of tillers/plant, number of fertile tillers/plant, panicle length, panicle weight, filled grains/panicle, total grains/panicle, filled grain percentage, 100 grain weight, total grain weight/plant, biomass, and harvest index were recorded according to the Standard Evaluation System (SES) for Rice. Days to flowering, was counted at 50% flowering. The data were statically analysed using the SPSS version 20 software.
(iv) Identification of morphological similarities and diversities of traditional rice cultivars with the same name by collecting data on plant height, tiller number, number of fertile tillers/plant, days to flowering, panicle length, 100- seed weight, seed length, seed weight, yield/plant.
(v) Identification of morphological diversities of rice cultivars with different names: Principal Component analysis (PCA), cluster analysis and morphological dendrograms using Ward Linkage were used to assess the patterns of the morphological variation. Principal component analysis sorted the accessions in to principal components that had within cluster similarities and inter cluster variations. Total variance was explained by the resulted principal components. At rescaled cluster distances, different rice accessions were clustered into different groups.
(vi) Identification of genetic diversity of traditional rice cultivars using SSR markers: More SSR markers are being added for the study.
(vii) Compilation of research data in to a book both in English and Sinhala: 2 books were already published.
(viii) Survey on traditional rice cultivars in remote areas of Sri Lanka: Data have been collected.
(ix) Publication of research findings: Publications were done based on morphological data.
(x) Training for a postgraduate student: An MPhil student is continuing the studies.
(xi) Financial assistance for page charges: Publications were done based on morphological data, publications based on molecular data will be published once the data collection is over.
(xii) Financial assistance for attending international conferences: The PI and a research student attended the Globe Research Forum, AIT, Bangkok, Thailand on 17-18 December 2015.
(xiii) Training on scientific writing: Finding on-site training was difficult since all the available courses were on-line. Finally, one scientist in Southern Queensland University agreed to allow me to attend the program in April 2016.

Results Unforeseen in the Original Project:

Training on technical writing.

URUGUAY

Title: Yeast mating as a model for cell-cell fusion

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Abstract: Fusion between cells underlies crucial events from fertilisation to the genesis of syncytial tissues. Although it has been studied for more than 150 years, the machineries that catalyse cell-cell fusion are still elusive. We use yeast mating as a model to uncover them. Our working model proposes that the protein Prm1 and extracellular Ca^{2+} regulate the fidelity of cell-cell fusion: in their absence cell-cell fusion turns into cell lysis. We exploited this model to perform genetic screens. Simultaneously, we performed bioinformatic searches using structural information of known fusogenic proteins. The results of these approaches lead us to develop a system to monitor cytosolic Ca^{2+} levels in single live cells and to describe, for first time in yeast, single cell Ca^{2+} dynamics with high temporal resolution. We also developed a high-throughput method to score cell fusion. Best candidates have been challenged by three rules we claim a true fusogen must follow:
(i) It should be necessary for cell-cell fusion;
(ii) It should localise at the site of cell-cell fusion and
(iii) It should be sufficient to promote cell-cell fusion when expressed in a heterologous system.
We have found a strong candidate, which is being currently challenged by rule number three.
Overall, this work pushes forward our knowledge on the molecular machineries that catalyse cell-cell fusion and the role cytosolic Ca^{2+} plays during this process.

Objectives:
(i) Identify genes that mediate cell lysis during mating in the absence of *PRM1*;
(ii) Identify genes that are structurally similar to known fusases;
(iii) Analyse the role of the identified genes in cell-cell fusion.

Results Obtained:
We identified genes involved in extracellular Ca^{2+} uptake during mating that mediate lysis and are linked to *PRM1*. We developed a novel system to monitor cytosolic Ca^{2+} levels in single live cells. Monitoring cytosolic Ca^{2+} levels during pheromone response in different genetic backgrounds by live microscopy enabled us to describe, for first time in yeast, single cell Ca^{2+} dynamics with high temporal resolution showing that cytosolic Ca^{2+} transients are not amplitude- but frequency-modulated. Two manuscripts are currently in preparation. Bioinformatics searches were performed using structural information of known fusogens. In order to count with a high-throughput method to score cell fusion during yeast mating we developed a novel flow cytometry- based cell-cell fusion assay. Best candidates have been challenged by three rules we claim a true fusogen must follow as indicated above.

Results Unforeseen in the Original Project:
The fusogen candidate prompted us to expand our work to a different model organism.

Publications:
Aguilar, P.S., Baylies, M.K., Fleissner, A., Helming, L., Naozaku, I., Podbilewicz, B., Wang, H-M., Wong, M. Genetic Basis of cell-cell fusion mechanisms. 2013. Trends Genet., **29**, 427-437
Zhang, S., Zheng, H., Long, N., Carbó, N., Chen, P., Aguilar, P.S., Lua, L. FigA, a putative homolog of low-affinity calcium system member Fig1 in *Saccharomyces cerevisiae*, is involved in growth and asexual and sexual development in *Aspergillus nidulans*. 2014. Eukaryot. Cell. **13(2)**, 295-303
Salzman, V., Porro, V., Bollati-Fogolin, M., Aguilar, P.S. Quantitation of yeast cell-cell fusion using multicolor flow cytometry. 2015. Cytometry A. **87(9)**, 843-854
Valansi, C., Moi, D., Leikina, E., Matveev, E., Graña, M., Chernomordik, L.V., Romero, H., Aguilar, P.S., Podbilewicz, B. *Arabidopsis* HAP2/GCS1 is a gamete fusion protein homologous to somatic and viral fusogens. 2017. J. Cell. Biol. **216(3)**, 571-581
Carbó, N., Tarkowski, N., Ipiña, E.P., Dawson, S.P., Aguilar, P.S. Sexual pheromone modulates the frequency of cytosolic Ca^{2+} bursts in *Saccharomyces cerevisiae*. 2017. Mol. Biol. Cell. **28(4)**, 501-510

VENEZUELA

Title: Study of genetic susceptibility in infectious diseases (Tuberculosis)
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Abstract: TB is an infectious disease caused by the bacillus *Mycobacterium tuberculosis*, which typically affects the lungs (pulmonary TB) but can affect other sites as well (extrapulmonary TB). This disease remains a major global health problem. In the WHO Global Tuberculosis Report for 2014 there were 9 million new TB cases in 2013 and 1.5 million TB deaths, of which 218,875 new cases and relapses occurred in the Americas. In Venezuela, the incidence of new and relapse TB cases is between 20 and 49 per 100,000 inhabitants. Following infection with the bacillus, some individuals completely contain and eradicate the organisms. Nevertheless, others develop latent infection and remain at lifetime risk for re-activation to active disease and others may manifest rapid clinical progression with dissemination shortly after becoming infected. Several lines of evidence suggest that host genetics strongly influences susceptibility to TB. The apparent variation between genetic backgrounds provides insights into the breadth of possible inflammatory responses. The association of host genetic factors with susceptibility or resistance to TB has been studied extensively, revealing several candidate genes involved in susceptibility. These studies have been performed in different ethnic groups, with large discrepancies between groups regarding the effect of the different candidate genes.
Objectives:
The aim of the present study was to characterise genetic variations associated with increased risk to a specific infectious disease (tuberculosis) and/or disease progression in order to permit the elaboration of drugs and treatments taking into account the host immune response to the infection.
Results Obtained:
The study was divided in three important aspects: (i) the association of genetic polymorphisms with susceptibility to pulmonary tuberculosis; (ii) the expression of genes associated with immune response and (iii) the gene expression microarrays (mice and human).
In relation to the association of genetic polymorphism with TB we performed the determination of variations of *NRAMP*, *VDR*, *NOS2*, *TNFA*, *IFNG*, *IL6*, *IL10* and *TGFB1* genes. The results suggest the absence of any association between *VDR* variants FokI, ApaI, and TaqI and susceptibility to tuberculosis. In contrast, the *NRAMP1* 3'UTR variants were associated with susceptibility to *M. tuberculosis* infection, as seen in the comparisons between TST+ and TST− controls, and also with progression to TB disease. On the other hand, our results showed not significant association between -954G/C *NOS2A* variant and susceptibility to either infection or the development of tuberculosis. However, the production of nitric oxide would control the *Mycobacterium tuberculosis* and the presence of other variants of *NOS2A* gene could determine its concentrations. Finally, there was no significant association between *TNFA* (-308 G/A), *IFNG* (+874 A/T), *IL10* (-819 C/T, -592 C/A) and *TGFB* (–869 T/C, –915 G/C) genotypes with TB. However, after Bonferroni adjustment, significant associations were detected between *IL10* and *IL6* polymorphism with tuberculosis (*IL10* -1082/AG and *IL6* -174/GC genotypes were negative or protective and *IL6*-174/GG and CC genotypes were positive or susceptible). In conclusion, the polymorphisms of *IL10* (-1082 G/A) and *IL6* (-174 G/C) genes could play a role in the protection or susceptibility to develop tuberculosis in the Venezuelan population. Also, several genes participate in the development of tuberculosis, and the pathogenesis of this infectious disease is the result of the interaction or combined effect of these genes. Therefore, further studies are needed to determine the role of genetic factors in the development of TB. With respect to expression of genes associated with immune response, human monocyte-derived macrophages infected with *M. smegmatis*, *M. smegmatis* + pYU18 (cosmid without *Mycobacterium tuberculosis* genes) or *Mycobacterium smegmatis* transformed with genes of *Mycobacterium tuberculosis* (pCEF) expressed pro-inflammatory cytokines such as IL-1A, IL-B and TNF-alpha. These cytokines are produced as a result of the microbicidal activity of the macrophage, but the macrophages infected did not express IL-6, IL-12 and enzyme NOS2. Also, culture supernatants of infected macrophages with the different conditions (*M. smegmatis, M. smegmatis + pYU18* or *M. Smegmatis + pCEF*) were negative for the Griess reaction, indicating the absence of nitrite due to the not induction of NOS2. This is because the nitric oxide production by activated macrophages requires IFN-gamma together with TNF-alfa for inducing the expression of the enzyme nitric oxide synthase. Finally, the study by microarrays of gene expression realised in mice showed genes expressed differentially.
Results Unforeseen in the Original Project:

The study by microarrays of gene expression in macrophages of mice infected with *M. smegmatis*, *M. smegmatis* + pYU18 or *Mycobacterium smegmatis* + pCEF showed genes expressed differentially (not down regulated, poorly regulated and equally up regulated) by *Mycobacterium smegmatis* + pCEF versus *Mycobacterium smegmatis* + pYU18.

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VIET NAM

Title: Mitogenomic analysis of neglected tropical parasitic heterophyid and paragonimid zoonoses for diagnostic and epidemiological application in Viet Nam

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Abstract: Mitochondrial DNA (mtDNA) is a source of useful markers for diagnosis, taxonomic identification/clarification/discrimination and epidemiological studies. During the project, the mtDNA sequences for three zoonotic trematodes, the small intestinal fluke, human *H. taichui* (15,119 bp, strain of Vietnam), the lung liverflukes, human *P. ohirai* (14,818 bp, Japanese origin) and human *P. heterotremus* (near complete, 13,527 bp, strain of Vietnam) were obtained and comparative analysed with other trematodes available to date. The organisation of these genomes is completely similar to those of trematodes in the Platyhelminthes phylum except African schistosomes. These mtDNA genomes contain 12 protein-coding, 2 ribosomal and 22 transfer RNA genes; and 2 non-coding regions (short, SNR and long, LNR), of which there are arrays of identical tandem repeats (TR) in the LNR. One gene (*atp8*) commonly found in other phyla is absent in these trematodes. Sequence overlapped is found between *nad4L* and *nad4* in all species, and unusually, in *Haplorchis taichui,* between *cox2* and *nad6.* The gene order has similarities to those seen in trematodes, although some exceptions are of transfer RNAs and tandem repeats in the con-coding region. Data from the complete mitochondrial genomes for *Haplorchis taichui, Paragonimus ohirai, P. heterotremus* support the clarification and taxonomic discrimination in Trematoda class (Heterophyidae family; Paragonimidae family) and in the Platyhelminthes phylum. Valuable mtDNA markers (such as *cox1, nad1, rrn*S,) were used to study these three trematodes and a single-step multiplex-PCR been developed for simultaneous detection of *Paragonimus heterotremus* and *P. ohirai.*

Objectives:

The major objective of this ICGEB project was to obtain and to annotate/analyse the (near)/entire mt genomes of common zoonotic parasitic species (three obtained: *Haplorchis taichui; Paragonimus ohirai; Paragonimus heterotremus*) in order to provide conserved sequences for PCR/LPCR primer, LAMP (loop mediated isothermal amplification), multiplex-PCR for further amplification/investigation/analysis of other platyhelminths and varied regions for diagnosis and systematics-based studies; as well as for the use of the resulting genetic markers to study molecular epidemiology of zoonotic parasites in Viet Nam and in other parts of the world of the relevant trematodes.

The original objectives were as follows:

(i) to obtain the complete (near) mt for *Haplorchis taichui;* with full annotation/characterisation; (ii) to obtain the complete (near) mt genome for a common lungfluke, *Paragonimus heterotremus,* from Vietnamese isolates, as optional work; (iii) to obtain nuclear markers (ITS-1 and/or ITS-2) for combination of analysis; (iv) to analyse genetic variability of mt genes/genomes and nuclear ITS for species for epidemiological study; (v) to develop a sensitive, one-step multiplex-PCR diagnostic kit(s)/protocols as duplex-PCR for two, or triplex-PCR for three species detection/discrimination; (vi) to develop a sensitive, one-step LAMP-based diagnostic kit(s) for different life-stage forms of these species from different hosts including humans, fishes, crabs and animals.

To complete these tasks, we would have to obtain: (i) the (near) entire mt genomes for at least 2 - 3 important species of public health concern; (ii) molecular annotation of the data and taxonomic identification/clarification; (iii) valuable genetic markers applicable for multiplex-PCR and/or LAMP performance; (iv) epidemiological study of these species; (v) additionally, the resulting data will provide directions for epidemiological control of increasing number of these zoonotic infections over the world.

Results Obtained:

Mitochondrial genomics: The mtDNA sequences for the small intestinal fluke, human H. taichui (**HtQT**, Quang Tri strain of Vietnam) (complete, 15,119 bp); for the lung liverflukes, human *P. ohirai* (**PoJP**, Japanese strain) (complete, 14,818 bp), and human *P. heterotremus* (**PhVN**, Lai Chau strain of Vietnam) (near complete, 13,527 bp) were obtained. The organisation of these genomes is completely similar to those of trematodes except African schistosomes in the Platyhelminthes phylum known to date. These mtDNA genomes contain 12 protein-coding, 2 ribosomal and 22 transfer RNA genes. One gene (*atp8*) commonly found in other phyla is absent from these flukes. The *nad4L* and *nad4* overlap each other by 40 bp at 3' end of *nad4L* and 5' end of *nad4* (in all three species) and unusually, overlapping sequence of 14 bp found at end of *cox2* and start of *nad6* in *H. taichui.* Transfer RNAs are arranged in the order of *trn*H; *trn*Q; *trn*F; *trn*M;

*trn*V; *trn*A; *trn*D; *trn*N; *trn*P; *trn*I; *trn*K; *trn*S1(AGN); *trn*W; *trn*T; *trn*C; *trn*L1(CUN); *trn*S2(UCN); *trn*L2(UUN); *trn*G; and *trn*E) in *H. taichui* but in paragonimids, *trn*G is downstream of *trn*E. With nearly 40 complete mtDNA genomes of platyhelminths available to date, the total length of protein-coding genes (calculation by adding of the sequence for concatenated 12 protein-coding genes) is around ~10.1-10.4 kb in the trematodes and 10-10.1 kb in the cestodes and others. The A+T usage for building up the protein genes, however, makes the platyhelminths dividing into different patterns: the schisotomes and tapeworms use of over 70% A and T while the heterophyids/paragonimids/fasciolids/opisthorchiids less, between 58-64% (around 62%). The least usage of A+T is among the paragonimids (58.8% in *P. heterotremus*; 51.6%, in *P. westermani*). In these three trematodes, the most frequent codon usage is of TTT-Phe, TTG-Leu and GTT-Val codons and the least is of CAA-Gln and CGC-Arg. All the three flukes in this study have a very long non-coding region arranged with tandem repeats (TR) and hairpin/loop structures. In mtDNA of *H. taichui*, repetitive region is located between *trn*E and *trn*G, containing three, TR1A, TR2A and TR3A of 182 bp (type A) and another three TR1B, TR2B and TR3B of 183 or 187 bp (type B) identical tandem repeats. In *P. ohirai*, downstream of *trn*G, there are two long TRs (termed as LTR1 and LTR2 of 292 bp/each) flanking *trn*E; and then followed by an array of 6 STRs (short tandem repeats of 117 bp/each). In *P. heterotremus*, a long non-coding region (of about 3 kb) consisting of multiple TRs was also found to link *trn*E with *cox3*, but was not able to be sequenced. Most tRNAs have standard "clover-leaf" structure with exceptions for Serine missing the DHU arm, *trn*S1(AGN) in *H. taichui* and both *trn*S1(AGN) and *trn*S2(UCN) in *P. ohirai* and *P. heterotremus*. Codonusage in the paragonimid species is similar to each other but slightly differs from that of *H. taichui*. As results, new mitochondrial genomic data for a range of zoonotic flukes, including these three common flukes of our study, provide a rich and valuable source of genetic markers for speciation/diagnosis/identification, biogeography, phylogenetics, variation/hybridisation of genotypes, population genetics, molecular epidemiology, and possibly, new insights into mitochondrial function for targeting drugs. Data from the complete mitochondrial genomes for *Haplorchis taichui, Paragonimus ohirai, P. heterotremus* support the clarification and taxonomic discrimination in Trematoda class (Heterophyidae family; Paragonimidae family) and in the Platyhelminthes phylum.

Molecular epidemiological studies: (i) Molecular identification of small intestinal flukes, *Haplorchis taichui* and *H. pumilio* collected in Vietnam, using mitochondrial *cox1*; and *16S (rrnL)* genetic markers, confirmed taxonomic characteristics and positions of these and a number of heterophyids; (ii) Molecular identification of a Vietnamese *Paragonimus* sample using mitochondrial *nad1* genetic marker revealed this species is *Paragonimus heterotremus*; (iii) Molecular identification and characterisation of the lung flukes, *Paragonimus heterotremus* and *P. ohirai* from the sample of Vietnam and Japan, respectively.

Multiplex-PCR development: A single-step multiplex polymerase chain reaction (PCR) has been developed for simultaneous detection of *Paragonimus heterotremus* and *P. ohirai* (as duplex PCR or commonly named as mPCR). These species may geographically overlap in distribution in China and maybe in North Vietnam. Morphological examination may result in confused conclusion due to similar egg size and shape. Based on a comparative alignment of mitochondrial DNA (mtDNA) spanning the ribosomal region of *rrnL-trnC-rrnS* (16S and 12S ribosomal DNA), two species-specific forward primers were designed on 12S gene, PHEF (for *P. heterotremus*) and POHF (for *P. ohirai*), and a single reverse PHOWR (common for both species). Testing reactions were applied to verify specificity of primers and template by conventional PCR followed by DNA sequencing. The mPCR (using 3 primers) was assayed to test with the DNA extracted from adult worms/eggs/metacercariae of these lungflukes, producing an amplicon of 598 bp for *P. heterotremus* and 433 bp for *P. ohirai*, respectively. The mPCR failed to amplify from DNA of other common liver and intestinal trematodes, including opisthorchiids, heterophyids, another fasciolid and possible respiratory microorganisms. The mPCR limit of detection for each *Paragonimus* species was between 0.02 ng and 0.01 ng DNA for each. This novel mtDNA mPCR (duplex reaction with three primers) is a sensitive and fast tool for accurate identification of *Paragonimus* spp in areas of distributional and zonal overlap.

During the period of the project several manuscripts were prepared for peer-reviewed publications in international journals and six papers published in national Vietnamese journals with English abstract available. Two MSc students (one thesis in English and one in Vietnamese) and two internship students completed their studies on the basis of the work carried out and data obtained with the support of this ICGEB grant. Additionally, exchange study and presentations were completed in Sri Lanka and Thailand and an International Workshop was organised with the aims of exchanging research results and training the participants in Vietnam.

All the above achievements were obtained in collaboration with: Prof. David Blair, James Cook University (Townsville, Australia); Prof R.P.V. Jayanthe Rajapakse, University of Peradeniya, Sri Lanka; Prof. Takeshi Agatsuma, Department of Environmental Health Sciences, Kochi Medical School (Kochi, Japan); Prof. Nguyen Van De, Hanoi Medical University (Viet Nam); Dr. Nguyen Thi Bich Nga, Ms. Nguyen Thi Khue, Ms. Do Thi Roan, Ms. Vu Thi Tien, all from the Institute of Biotechnology (Viet Nam).

Results Unforeseen in the Original Project:
We have expected to obtain mt genome for one heterophyid (*H. taichui*) and one paragonimid (*P. heterotremus*) but in fact, additionally, the complete mtDNA for *P. ohirai* was successfully sequenced. We have faced with sequencing tandem sequence repetitive non-coding region in all three species that slowed down the achievements for publications in due time but soon will be available. The findings of repeats confirmed the polymorphic features in trematodes.

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