

Final Report /

Conservation International MMAS Award: Advanced BioSensors 2.1.5 “Development of genomic and molecular assays for identifying environmental stress responses in the coral *Pocillopora damicornis*”

Addendum: “Application of high throughput sequencing and DNA microarray analysis to the identification of stress-response genes in the coral *Pocillopora damicornis*”

SUMMARY of FINDINGS

Overall, we were able to accomplish significantly more than we initially proposed. We can attribute this unanticipated degree of success to four factors: (1) we benefited from technological advances in DNA sequencing (and concomitant price reductions) that occurred in the last few years; (2) we were able to pursue unanticipated promising findings, including the discovery of a *grainyhead* gene (which is associated with wound healing in higher animals) in cnidarians; (3) the project attracted additional collaborators; (4) the preliminary findings were sufficient to leverage additional funding from NSF and Boston University (and a new grant proposal to extend this research is currently pending at NIH).

RESULTS OF EACH SPECIFIC AIM IN THE ADDENDUM

Below, each specific aim from the addendum (boxed text) is followed by the results we obtained.

I. “Isolation of RNA from stressed and unstressed corals. [April-May, 2009] We will isolate a diverse pool of RNA from stressed and unstressed coral tissue from different source populations. RNA isolation will be performed in April and May of 2009 by Nikki Traylor-Knowles, a 4th year PhD candidate who is co-mentored by Drs. John Finnerty and Les Kaufman (Boston University). Tissue samples from stressed and unstressed corals will be provided by Dr. Bob Richmond (U. Hawaii). Stressed corals will be exposed to acute temperature stress, low pH, peroxide, and UV-stress. Coral tissues will be obtained from multiple geographically discrete coral populations identified by Bob Richmond.”

RESULTS: Ms. Traylor-Knowles traveled twice to Hawaii in 2009, once in May and once in October. She obtained live *Pocillopora damicornis* from Dr. Bob Richmond. The corals were collected from three geographically distinct populations (Figure 1) in locales experiencing differing degrees of human impact.

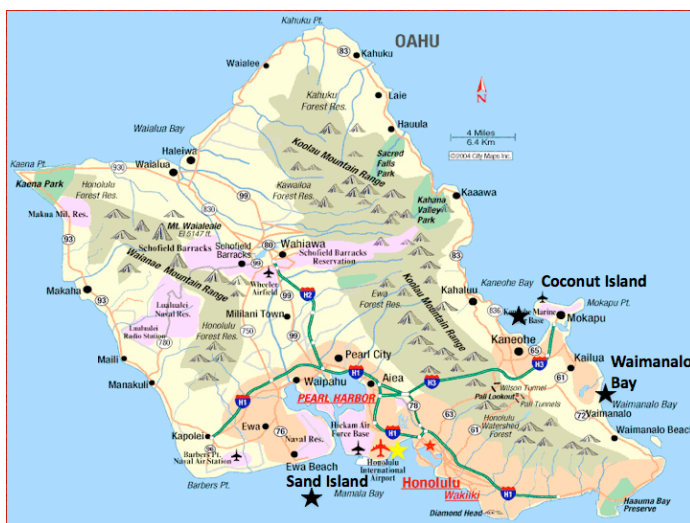


Figure 1. Map of Oahu showing the three sites from which *P. damicornis* were obtained (black stars). Sand Island is a shallow, calm, bay densely populated with *P. damicornis*. It is adjacent to an industrial site. Waimanalo is a public beach, subject to heavy wave action. *P. damicornis* is quite rare in this location. The Coconut Island site is a sheltered lagoon, where *P. damicornis* is sparse.

Nubbins from each population were subjected to one of the following stressors: hydrogen peroxide (exposure to 10% H₂O₂ for 1 hour); heat shock (exposure to 50°C for 1 hour); desiccation (removal from water for 4 hours); ultraviolet light (exposure to 254 nm UV on a light box for 4 hours); hyposalinity

(exposure to fresh water for 2 hours). RNA was isolated from stressed and unstressed animals from all three populations. RNA of high quality was obtained (“260/280 ratio” > 1.8 and no evidence of degradation on a denaturing gel).

II. “High throughput sequencing of the coral transcriptome. [June-July 2009] We will forward 15 micrograms of the pooled RNA to Agencourt Biosciences (Beverly, MA). As per our agreement with them, Agencourt will construct a normalized library and produce ~1.2 million sequencing reads, averaging 400 nucleotides in read length, using their Titanium PicoTiterPlate™ Pyrosequencing platform. This will yield >400 million nucleotides of sequence data, which we estimate to be >16 times greater than the cumulative length of the entire *Pocillopora* transcriptome. Agencourt will assemble the sequence data into contigs and generate consensus sequences for every transcript. The assembled and raw sequencing data will be transferred to Boston University via ftp. From the delivery of RNA (target date June 1) to the completion of the sequencing will require 8 weeks.”

RESULTS: The initial attempt to sequence the *P. damicornis* transcriptome in summer of 2009 failed due to degradation of RNA samples attributable to mishandling by Agencourt Biosciences (now subsumed within Beckman/Coulter Genomics). The subsequent RNA sample supplied by us in October of 2009 was successfully sequenced. The data were delivered to us on January 10, 2010. In total, 1,116,553 sequencing reads were generated. Of these, 955,105 were assembled into 70,786 “contigs” with an average length of 836.24 nucleotides.

III. Bioinformatic analysis of coral genes. [August-September, 2009] We will provisionally characterize the orthology of all genes using BLAST searches, and we will organize these data into a searchable database (akin to StellaBase, which we developed to house genomic data from the sea anemone *Nematostella*; www.stellabase.org; [1])

RESULTS: Upon receipt of the 454-sequencing data in January, 2010, Ms. Traylor-Knowles commenced an initial homology screen of the sequences. As hoped, this screen identified many of the stress-response genes we were seeking to obtain, including members of the NF- κ B stress-response network and the transcription factor *grainyhead* (Grh), which is associated with wound healing. This validates our approach, and gives us confidence that a thorough bioinformatic analysis of these data will uncover a complete or nearly complete repertoire of stress-response genes from the coral. The results of a homology analysis where the coral sequences were compared against the human genome are appended in an excel spreadsheet. In ongoing analyses, we are parsing the sequences into a number of taxonomic categories, including the following: genes which are shared with “higher animals” genes which appear unique to Cnidaria; genes which appear unique to *Pocillopora*; genes of suspected zooxanthellae origin; genes of other microbial origin; putative viral genes; putative fungal genes. We are also categorizing the genes by gene ontology (GO) terms, including genes that are associated with various cellular stress responses. As described below, the data will soon be accessible via a searchable, user-friendly web interface.

To complete our bioinformatics analysis, we have recruited a number of additional researchers interested in coral conservation genomics, including several computational specialists. These include Tristan Lubinski and Derek Stefanik, PhD candidates from the Finnerty lab, as well as Brian Granger and Sarah Garamzegi, first-year students in BU’s Bioinformatics graduate program. Thus, the CI investment is leveraging a significant investment of new intellectual capital, and I’m hoping that one or more of these individuals will continue to work on coral-stress genomics going forward. The short-term objective of this collaboration is to develop a public searchable database akin to StellaBase—The *Nematostella vectensis* Genomic Database. We will announce the availability of the Pdam database to the coral community as soon as it is functional [by early summer 2010].

IV. Establishment of a “coral farm” at Boston University. [April-December 2009] We will construct and populate a *Pocillopora damicornis* culture facility sufficient to supply all the required coral for the

proposed stress-response experiments, in addition to accommodating a limited number of requests for live coral or coral tissue from the larger community of coral researchers (see “Deliverables” below).

We have established a coral culture facility in the Biology Research Building at Boston University. It is designed to house 150-210 *P. damicornis* fragments. It is a 200-gallon system comprising 3 50-gallon holding tanks, ~250 pounds of live rock, and ~200 pounds of sand. We have awaiting the imminent delivery of corals from Panama (through Tom Capo) and from Palau (through Bob Richmond).

V. Analysis of stress-response gene expression patterns. [October-December 2009] We will work with Nimblegen to design a gene expression microarray for *Pocillopora damicornis*. The microarray will represent every gene identified in our high throughput sequencing project with an average of four different antisense oligonucleotides. Once designed and produced, the array will be available from Nimblegen for long term use by other coral researchers for a modest cost. Using this microarray, we will compare RNA samples from unstressed corals as well as those exposed to high temperatures, low pH, high ultraviolet light, and high levels of peroxide. This will identify genes that are specifically up-regulated in response to these particular stressors. This will allow us to develop a molecular diagnosis of coral stress, and it will give us insights into how the corals are mobilizing their cellular defense systems to counteract stressful environments.

Instead of developing a gene expression microarray for *P. damicornis*, we are using a high-throughput DNA sequencing platform—an Illumina Genome Analyzer Iix—to directly sequence mRNA from animals subjected to control conditions or particular stressors. This approach is both more reliable (because it doesn’t require hybridization) and less expensive per sample, allowing us to analyze a greater number of RNA samples. The sequencing is being performed in the laboratory of a new Boston University collaborator, Professor James Galagan, in the department of Biomedical Engineering. Ms. Traylor Knowles has been working with Chris Mawhinney, a technician in Dr. Galagan’s lab, to optimize the preparation of our RNA samples for sequencing. We are “in the queue,” and expect that our samples will be sequenced in the next several weeks.

Aims of the original proposal

As discussed in the addendum, some of the original aims have been accomplished, some have been subsumed into the new genomic approach, and some are not yet complete but are continuing unabated. Here, I describe some **NEW FINDINGS** that relate specifically to these original aims.

(1) To clone and characterize the genes in the canonical NF-κB pathway (NF-κB, NF-AT, Bcl3, IκB, and Toll receptors) from the coral *Pocillopora damicornis*.

RESULTS—As described in an earlier progress report, we succeeded in cloning and sequencing NF-κB from two corals: *Pocillopora damicornis* and *Acropora cervicornis* and from another sea anemone, *Aiptasia pallida*. Using computational methods, we had also succeeded in identifying both NF-κB and IκB from published EST data from the coral *Acropora millepora*. As discussed in the addendum, we suspended our gene-by-gene search for NF-AT, Bcl3, IκB, and Toll receptors because we expected these genes would be retrieved in our “454” transcriptome sequencing project, and searching for them directly would slow down the transcriptome sequencing. **NEW FINDINGS:** We are pleased to report that the transcriptome sequencing project was enormously successful, and while we have only been able to computationally analyze a small fraction of the data, we did indeed recover numerous genes in the canonical NF-κB pathway from the coral *Pocillopora*. **When the full analysis is complete, we expect that we will recover the entire NF-κB stress-response pathway.**

(2) To develop antibodies for the NF-κB, NF-AT, Bcl3, and IκB proteins in *P. damicornis*.

- (3) **To monitor the localization and function of the NF- κ B pathway of *P. damicornis* in response to abiotic stressors (oxidative stress, temperature stress) and pathogen exposure (viral, bacterial, fungal). The following specific questions will be addressed.**

CHANGE IN DIRECTION—We had previously focused on the NF- κ B pathway because we believed it to be an inroad into the stress response repertoire of corals because it serves as a general regulator of stress in “higher animals.” However, as discussed in the addendum, we switched to a functional genomics approach because it is a more direct route to characterizing the entire molecular stress response of the coral. We therefore applied the remaining funds from the original grant and refocused our research efforts on a functional genomics approach (described above).

However, at the same time, we accelerated our work on NF- κ B with support from a new 3-yr grant from the National Science Foundation. Our recently published paper on a NF- κ B polymorphism in *Nematostella* [2] proves that variation in NF- κ B can partially explain why individuals differ in their stress tolerance. The high-throughput sequencing project will utilize RNA isolated from geographically distinct *P. damicornis*, and therefore maximize our opportunity to detect different NF- κ B variants in the coral. Additionally, the high-throughput sequencing should uncover all of the genes that comprise the NF- κ B signaling pathway and those genes that are targets of NF- κ B regulation much faster than we could using piecemeal cloning approaches.

EXTENSION OF CI FUNDED RESEARCH. As part of our collaboration with the Gilmore lab, we have successfully produced **an antibody to the *Nematostella* NF- κ B protein**. This antibody specifically recognizes the *Nematostella* protein (in a western blot), and Francis Wolenski in the Gilmore lab has recently shown that it labels the *cytoplasm* of a class of abundant but scattered cells in the outer epidermal layer of the animal. We are proceeding to characterize these cells, which may represent a kind of cnidarian stress response or immune cell, a finding which would have enormous impact. To validate this hypothesis, we are determining whether exposure to particular stressors causes the NF- κ B protein to move from the cytoplasm to the nucleus of these cells, a key step in the process whereby NF- κ B “turns on” stress-response genes. We will continue to pursue the development of antibodies to other NF- κ B signaling proteins in our ongoing collaboration with the lab of Tom Gilmore using NSF support.

- (4) **To identify and clone stress-response and pathogen-response genes in *P. damicornis* using experimental and computational studies on the closely related genomic model system *Nematostella vectensis*.**

RESULTS As discussed in the addendum, we investigated the effects of thermal stress, oxidative stress, and salinity stress on the growth, regeneration, and gene expression of the sea anemone *Nematostella*.

(1) Thermal stress. Both organismal growth (a) and regeneration rate (b) were monitored at steady-state conditions of 13°C, 21°C [the normal culture temperature], and 29°C. The effects of heat shock and cold shock on regeneration were also evaluated (c), and RNA samples were isolated from temperature-shocked animals so that gene expression profiles can be evaluated (d). As described in our previous progress report, we monitored growth and regeneration of clonal lines generated from wild populations of *Nematostella* collected along the Atlantic coast of North America from Kingsport, Nova Scotia, to Baruch, South Carolina. We demonstrated that temperature had a pronounced and highly significant effect on polyp growth, but importantly, clone lines from different estuaries differed significantly in their temperature specific growth rates. In sum, we succeeded in identifying **genetically based differences in temperature-specific growth rates** in the sea anemone. We also found that temperature had a pronounced and highly significant effect on the rate of polyp regeneration (the first such study of which we are aware that utilizes regeneration rate of individual polyps as an organismal measure of environmental adaptation. More importantly, we were also able to identify **significant differences among clone lines in**

their temperature-specific regeneration rate. These results are described in a manuscript currently in preparation.

(2) Oxidative Stress. As described in a previous progress report, hydrogen peroxide (0.167 micromolar) was found to have a significant deleterious effect on the regeneration success rate. Importantly, **the effect differs dramatically between individuals based on their genotype at the NF- κ B locus.** Animals that were homozygous for a cysteine residue (C/C) at position 67 of the Rel homology domain were unable to regenerate in the presence of peroxide (n=7). By contrast, four of seven animals that were homozygous for a serine residue (S/S) at the same position were able to regenerate successfully. Thus, **we have identified genetic differences in the ability to resist oxidative stress.** Additionally, working with our collaborators in the Gilmore lab, **we characterized numerous differences in the molecular function of the two native proteins (the “Cys” variant and the “Ser” variant).** Based on electrophoretic mobility shift assays (EMSAs), the two proteins differ in their ability to bind DNA under normal conditions and when thiol-containing contaminants are added to the water (as frequently occurs via natural or anthropogenic means in coastal marine habitats). The two alleles also differ in their ability to drive transcription of a reporter construct in cell culture. **RECENT PUBLICATION: These results were recently published in PLoS ONE [2].** When the transcriptome sequencing data are fully analyzed, we will determine whether a similar polymorphism exists in the Hawaiian *Pocillopora* we have sampled.

(3) Salinity Stress. Regeneration rate was monitored in animals exposed to salinity shock (hyposaline or hypersaline environments in step-wise increments involving 10 ppt shifts over a period of 48 hours). The following six steady-state salinities were compared (1 ppt, 11ppt, 21 ppt, 31 ppt, 41 ppt, and 51 ppt). *Nematostella* proved unable to regenerate at 1ppt, even though the animal has been reported at such low salinities, and its regeneration success was significantly retarded at 51 ppt versus 11-41 ppt. RNA was isolated from whole animals 1 hour and 4 hours after each salinity shift for subsequent gene expression analysis. This will allow us to identify the gene expression profile of a salinity-stressed animal. A rough draft manuscript has been prepared, but submission to a journal is not yet imminent.

NEW FINDING Interestingly, we have observed a pronounced difference in the tolerance of different individuals of Panamanian *P. damicornis* to acute hyposalinity shock. In a pilot experiment, in response to a temporary pronounced drop in salinity, some corals in our lab culture are unaffected while others experience a partial die-off. We are currently fragmenting and propagating both the hyposaline-resilient and hyposaline-susceptible corals so that we can begin to explore the possible molecular/genetic basis for this difference.

Role of addendum research in the larger Coral Whisperer program

[text carried over from the prior progress report]

The Coral Whisperer consortium encompasses five laboratories and three initial coral-symbiont systems: *Pocillopora damicornis*, *Acropora hyacinthus*, and *Montastrea faveolata*. Three laboratories are working principally with *P. damicornis*, as this is the crossover taxon between field and laboratory studies. **The sequenced transcriptome from pooled stressed *P. damicornis* will serve as a reference against which RNA isolated from laboratory-stressed corals (initiated under this addendum) and field-caught corals (in future work) can be compared.** As described above, the *P. damicornis* transcriptome should encompass complete or near-complete stress pathways and not just single markers, leading to greater potential for discriminating among candidate stressors, alone or in combination, in a differential diagnosis of coral health from particular sites. This addendum will also make it possible to bring the *P. damicornis* work nearly to par with the parallel efforts on *A. hyacinthus* now being pursued in the Palumbi laboratory.

Deliverables

Peer reviewed publications in print or in press.

1. Reitzel AM, Sullivan JC, Traylor-Knowles N, Finnerty JR: Genomic survey of candidate stress-response genes in the estuarine anemone *Nematostella vectensis*. The Biological Bulletin 2008, 214(3):233-254.
2. Sullivan JC, Wolesski FS, Reitzel AM, French CE, Traylor-Knowles N, Gilmore TD, Finnerty JR (2009) Two alleles of NF- κ B in the sea anemone *Nematostella vectensis* are widely dispersed in nature and encode proteins with distinct activities. PLoS ONE. 4(10): [e7311](#).
3. Traylor-Knowles N, Hansen U, Dubuc TQ, Martindale MQ, Kaufman L, Finnerty JR (2010) The evolutionary diversification of LSF and Grainyhead transcription factors preceded the radiation of basal animal lineages. BMC Evolutionary Biology. *In press*.

Scientific manuscripts in preparation.

4. Reitzel AM, Chu T, Edquist S, Genovese C, Finnerty JR (in prep) Differences in growth rate and regeneration rate imply local adaptation to temperature in populations of the estuarine sea anemone *Nematostella vectensis*.
5. Dubuc T, Traylor-Knowles N, Hansen U, Finnerty JR Martindale MQ (in prep) Wound healing in the sea anemone *Nematostella vectensis* requires MAPK/ERK signaling and is associated with grainyhead expression.
6. Lubinski T, Granger B, Opfelt S, Stefanik D, Traylor-Knowles N, and Finnerty JR (in prep) The *Pocillopora damicornis* genomics database.
7. Wolenski F, Traylor-Knowles N, Lubinski T, Finnerty JR, Gilmore TD (in prep) Insights into the ancestral NF κ B signaling-pathway from the sea anemone *Nematostella vectensis* and the lace coral *Pocillopora damicornis*.

Related grants awarded or in review.

2009-2013

“Rel Homology Domain Signal Transduction Pathways in the Sea Anemone *Nematostella vectensis*.” [co-PI with Tom Gilmore; NSF MCB-[0924749](#); Total Award: \$573,015].

2009-2010

“Possible Functional Diversification of the CP2 and p53 Protein Families from a Common Ancestor Early in Animal Evolution—Evidence from the Basal Animal Model *Nematostella vectensis*.” [PI, with fellow-PIs Ulla Hansen and Zhi-Xiong Jim Xiao; Genome Science Institute, Boston University; Total Award: \$10,000].

Proposal in Review

“Improved genetic methods and molecular tools to analyze basic and applied biological processes using an emerging model organism, the starlet sea anemone *Nematostella vectensis*.” [co-PI with Tom Gilmore and Cynthia Bradham; National Institutes of Health; Total Request: \$448,455].

Additional anticipated publications based on findings generated by the grant.

1. Salinity challenges. A paper detailing the effects of varying steady-state salinities and applying salinity shocks to regeneration in the sea anemone *Nematostella* and survival/gene expression in the coral *Pocillopora*. **Summary of findings:** Regeneration rate was monitored in animals exposed to salinity shock (hyposaline or hypersaline environments in step-wise increments involving 10 ppt shifts over a period of 48 hours). The following six steady-state salinities were compared (1 ppt, 11ppt, 21 ppt, 31 ppt, 41 ppt, and 51 ppt). *Nematostella* proved unable to regenerate at 1ppt, even though the animal has been reported at such low salinities, and its regeneration success was significantly retarded at 51 ppt versus 11-41 ppt. RNA was isolated from

whole animals 1 hour and 4 hours after each salinity shift for subsequent gene expression analysis. This will allow us to identify the gene expression profile of a salinity-stressed animal. As discussed above, in *P. damicornis*, we have already noted an apparent difference in tolerance of hyposaline conditions, and a freshwater challenge is one of the stressors whose impacts on gene expression we are investigating using Illumina sequencing.

2. Comparative genomics studies. The sequencing of the *Nematostella* genome spawned several dozen publications in the two years subsequent to its release, and the rate of new publications appears to be increasing. The sequencing of the *Pocillopora* transcriptome should stimulate a comparable flurry of publications in the field of comparative cnidarian genomics beginning in early 2010 and continuing for several years thereafter.
3. Wound-healing gene expression studies. We are using Illumina Genome Analyzer (see above) to sequence mRNAs from *N. vectensis* and *P. damicornis* that are undergoing healing and regeneration of polyps following experimental resection.

Other deliverables.

Coral genomics database. We will generate a coral genomics database that will be accessible via a web interface. It will follow the model of StellaBase—The *Nematostella* genomics database (www.stellabase.org; [1, 3]).

Living clonal stocks. With support from CI, we have already identified wild caught strains of *Nematostella* that differ in their ability to tolerate temperature variation and peroxide. We are culturing these strains and building up their numbers so that we can accommodate requests for these particular animals from other cnidarian researchers. The research proposed in the addendum will allow us to develop laboratory stocks of *Pocillopora*, and these stocks may also exhibit variation in their stress tolerance, making them invaluable tools for understanding the evolution of resilience. These stocks will also be made available to other coral researchers, as soon as sufficiently large stocks are attained.

Field sampling kits. Informed by Nikki's field experience in Hawaii (May and October 2009), we are developing a protocol for our collaborator Bob Richmond to use in obtaining coral tissue samples for subsequent high-throughput mRNA sequencing. We expect the kits will consist of aliquots of "RNA-later" dispensed into 50-milliliter polypropylene tubes. Such kits would be small and lightweight and could be shipped at room temperature. We will also develop a coral sampling protocol sheet and field-data recording sheet that will be user friendly for non-scientists. This work will be completed after we have completed the bioinformatics analysis of the sequenced stress-RNA pools, so that we are confident that our tissue handling is not compromising our recovery of RNA sequences.

Stress-test kits for the classroom. I have initiated discussions with K-12 teachers in Massachusetts, North Carolina, Pennsylvania, and Texas to develop and deploy inexpensive instructional kits that would allow teachers to run science labs investigating the effects of various stressors on the regeneration rate of *Nematostella*. I am also developing accompanying instructional materials that will highlight the plight of corals and the relationships of sea anemones to corals. These materials will acknowledge the role of CI in funding coral conservation research.

I have also established a collaboration with Jacqueline W. Brittingham, the chair of the Biology Department Chair at Simpson College in Indianola, Iowa, to develop a research-oriented curriculum for undergraduates using *Nematostella* stress-regeneration assays and RNA isolation. In parallel with ongoing research in my lab, students at Simpson would study how particular stressors impact the ability of *Nematostella* to regenerate. They would also isolate RNA from the stressed anemones. We would then sequence the Simpson samples here at

BU and assist them in the analysis of the data.

LITERATURE CITED

1. Sullivan JC, Ryan JF, Watson JA, Webb J, Mullikin JC, Rokhsar D, Finnerty JR: **StellaBase: the *Nematostella vectensis* Genomics Database**. *Nucleic Acids Res* 2006, **34** (Database issue):D495-499.
2. Sullivan JC, Wolenski FS, Reitzel AM, French CE, Traylor-Knowles N, Gilmore TD, Finnerty JR: **Two alleles of NF- κ B in the sea anemone *Nematostella vectensis* are widely dispersed in nature and encode proteins with distinct activities**. *PLoS ONE* 2009, **4**(10):e7311.
3. Sullivan JC, Reitzel AM, Finnerty JR: **Upgrades to StellaBase facilitate medical and genetic studies on the starlet sea anemone, *Nematostella vectensis***. *Nucleic Acids Res* 2008, **36**(Database issue):D607-611.