

Pathogenic clones or environmentally determined population expansion? A molecular and epidemiological analysis of an epidemic of the human fungal pathogen *Coccidioides immitis*

Matthew C. Fisher and John W. Taylor, Department of Plant and Microbial Biology, University of California at Berkeley Email:mfisher@nature.berkeley.edu
Gina L. Koenig and Thomas J. White, Roche Molecular Systems, Alameda, California

ABSTRACT

A major epidemic of coccidioidomycosis occurred between 1991 and 1994 in central California.

Molecular analyses showed (i) **extensive genetic diversity**, (ii) **a lack of linkage disequilibria** and (iii) **little phylogenetic structure** demonstrating that a newly pathogenic strain was not responsible for the observed epidemic.

Epidemiological analyses showed that *C. immitis* morbidity was best explained by two variables, the length of droughts preceding epidemics and the amounts of rainfall.

This shows that the principle factors governing this epidemic of *C. immitis* are environmental and not genetic.

CONCLUSION: Long term environmental factors regulate the population size of *C. immitis* and are instrumental in determining sizes of epidemics. This knowledge provides an important tool for predicting outbreaks of this infectious disease.

METHODS

• Polymorphic Genetic Loci

Single nucleotide polymorphisms were found by amplifying arbitrary loci in a radioactive PCR to reveal single strand conformational polymorphisms (SSCPs) (9). 13 loci were subsequently scored by restriction endonuclease assays. All isolates were also typed for two loci containing polymorphic short tandem repeats (STRs), locus *621r* (containing an (AC)*n* (*n* = 6 – 18) microsatellite) and locus *B34* (containing a (TAA ACA AAC)*n* (*n* = 1 – 6) minisatellite) (10).

• Genetic Analyses

Population genetic analyses.

The disequilibrium coefficient between alleles of 78 pairs of loci were calculated. The index of association (IA) measures the degree of association between all loci based on the variation of the genetic distance between individuals (11,12) and its significance was assessed by comparison with values calculated from 1000 artificially recombined datasets (9).

Phylogenetic analyses. Maximum parsimony was used to find the shortest tree that fitted the data using PAUP 4.0 and the strength of branches was examined by bootstrapping. In order to test whether the observed dataset contained more phylogenetic signal than a population undergoing complete recombination, 500 artificially recombined datasets were created and the lengths of their most parsimonious trees compared to those found for the observed dataset (The 'Archie' test, 13).

• Epidemiological Analyses

C. immitis morbidity data was used for the period 1955 to 1995 in Kern County. To account for changes in the population size over time, the data was transformed by calculating the relative change in *C. immitis* morbidity (RCM) as the numbers of cases in year *n* divided by the numbers of cases in year *n* - 1.

Generalized linear models were constructed with RCM as the dependant variable and the following as independent variables:

- (A) Mean annual rainfall,
- (B) The occurrence of type I El Niño Southern Oscillation (ENSO) events
- (C) The annual Palmer Drought Severity Index (PDSI)
- (D) The length of moderate to extreme droughts preceding any particular year
- (E) Mean annual temperature
- (F) Mean annual 10 micron particulate matter concentration (PM10)
- (G) Mean annual total suspended particle concentration (TSP)
- (H) Yearly 1970 – 1996 Kern County population size.

Type I ENSO events (B), are known to be the principal determinants of above-normal rainfall in California and are defined as an equatorial Pacific sea surface anomaly of +2.0°C (Figure 1). Droughts (D) are defined as having a PDSI of -1 to -2.9 for nine months or more.

The fit of each linear regression model was assessed by inspection of the regression *r* values with their associated residuals, and tested with the F-statistic.

RESULTS

Genetic Analyses

- Alleles showed all epidemic-associated isolates to be members of the 'California' species of *C. immitis* (Figure 2).
- Multilocus genotypic diversity was high - 34 unique genotypes were observed in the sample of 37 epidemic isolates. Only 4/78 pairs of loci showed significant linkage disequilibria. The index of association was not significantly greater than that expected for a fully recombined population (*P* = 0.28).
- The strict consensus of 46, 072 maximum parsimony trees (Figure 2) is poorly resolved with few internal branches and most isolates falling into a polytomy of 31 isolates. Comparing the observed tree length against those from artificially recombined datasets showed no significant difference.
- Three pairs of isolates had identical genotypes (Figure 2). One pair (isolates 2005 and 2267, genotype S) was unlikely to be observed in this dataset (binomial probability; *P* < 0.001) and could be considered a genetically identical clone.

Epidemiological Analyses

- Multivariate regression found significant correlation between RCM and variables (A) **mean annual rainfall** and (D) **length of drought** (multiple *r* = 0.673; *P* (rainfall) < 0.01, *P* (drought) < 0.001).
- 45% of the variation in the yearly series is predicted by the regression equation $y = 0.295x_1 + 0.001x_2 - 0.16$ (x_1 = 'length of drought' x_2 = 'rainfall') and successfully predicts the 1991-1994 epidemic (Figure 4).

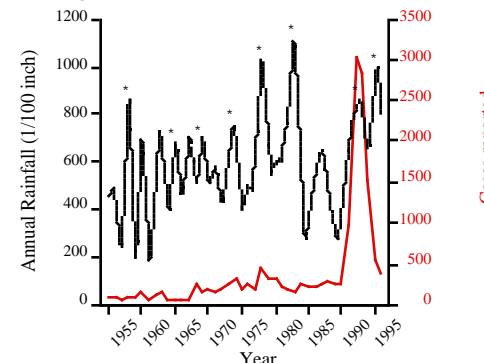


Figure 1 Annual number of cases of coccidioidomycosis reported from Kern County (red line) and annual rainfall (solid line). El Niño years are marked with an asterisk (*). Data courtesy of California Department of Health Services, Division of Communicable Disease Control.

CONCLUSION

- **The '91-'94 epidemic of coccidioidomycosis is attributable to environmental, and not genetic, causes.**

For unlinked loci, the medium time to genetic equilibrium in a randomly mating population with a recombination rate (*c*) of 0.01 is 69 generations and for *c* = 0.001, about 693 generations (14). Given that the generation time in *C. immitis* is at the most 1 - 2 per year we would not expect genetic equilibrium to be established within the time scale of this epidemic.

This rules out the evolution of a virulent *C. immitis* done as a cause of the 1991 - 1994 epidemic.

There is also direct evidence for clonal propagation, and subsequent infection, by *C. immitis* within this dataset. Two patients were infected by *C. immitis* with identical multilocus genotypes, isolates 2005 and 2267. The patients who contributed these isolates lived and worked in the same town, but the *C. immitis* isolates were collected by separate clinicians.

These people had been infected by the local dispersion of asexually produced *C. immitis* spores from a single fungal individual.

- **This analysis found the first statistical evidence to link rainfall, and its timing, with numbers of cases of coccidioidomycosis.**

Significantly, the drought preceding the 1991 - 1994 epidemic was the most sustained since 1956 implicating it in the development of this epidemic. How the length of droughts may effect the growth of *C. immitis* is a matter for conjecture. It is known that *C. immitis* is a poor competitor on nutrient media and is easily overgrown by common soil fungi, however is resistant to drought and high temperatures (15). We can speculate that this particular extended drought suppressed fungal competitors relative to *C. immitis* to the extent that constraints on the growth of *C. immitis* were released when the 1992 ENSO increased rainfall in California. We conclude that a fortuitous conjunction of climatic variables appears to have allowed the epidemic to occur.

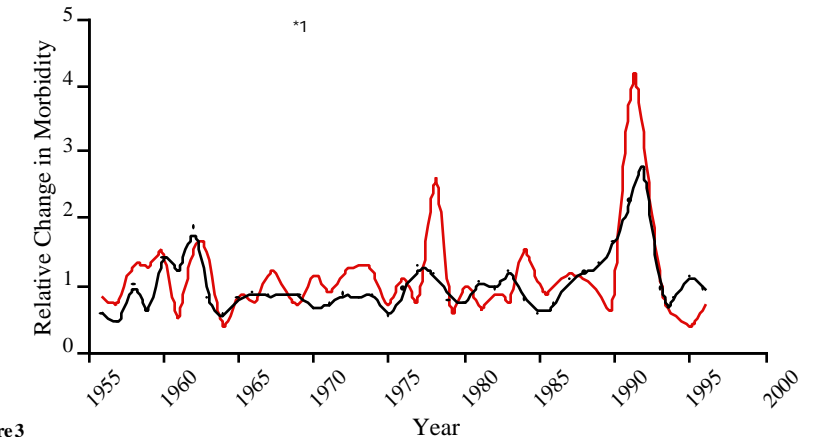


Figure 3 The relative change (RCM) in numbers of cases of coccidioidomycosis for Kern County during the time period 1956 - 1996 (RED LINE). The BLACK LINE shows the modeled change using the regression equation $y = 0.295x_1 + 0.001x_2 - 0.16$ with the variables x_1 = length of drought and x_2 = mean annual rainfall. *1 signifies an outlier data point.

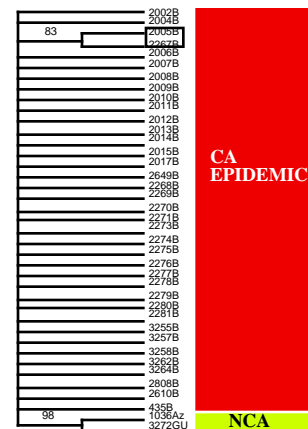


Figure 2.

Strict consensus of 46 072 most parsimonious phylogenetic trees. *C. immitis* isolates with identical genotypes are boxed. 'Outgroup' taxa from the non-California (NCA) species are shown in the green box.

REFERENCES

1. Geiser, D. M., Taylor, J. W., Ritchie, K. B. & Smith, G. W. Cause of sea fan death in the West Indies. *Nature* **394** 137-138 (1998).
2. Berger, L., Speare, R., Daszak, P. & Green, D. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *PNAS* **95**, 9031-9036 (1998).
3. De Sousa, A., Sanches, S., Ferro, M. L., Vaz, M. J. & De Lancastre, H. The Intercontinental spread of a drug-resistant methicillin-resistant *Staphylococcus aureus* clone. *J Clin Microbiol* **36**, 2590-2596 (1998).
4. Edlin, B. R. et al. An outbreak of multidrug-resistant tuberculosis among hospitalized patients with the acquired immunodeficiency syndrome. *N E J M* **326**, 1514 - 1521 (1992).
5. Karaolis, D. K. R. et al. A *Vibrio cholerae* pathogenicity island associated with epidemic and pandemic strains. *PNAS* **95**, 3134-3139 (1998).
6. Brasier, C. M., Cooke, D. E. L. & Duncan, J. M. Origin of a new *Phytophthora* pathogen through interspecific hybridization. *PNAS* **96** 5878-5883 (1999).
7. Arsura, E. et al. *Coccidioidomycosis* (eds. Einstein, H. E. & Catanzaro, A.) (National Foundation for Infectious Diseases, Washington D. C., 1996).
8. Burt, A. et al. Molecular markers reveal differentiation among isolates of *Coccidioides immitis* from California, Arizona and Texas. *Mol Ecol* **6**, 781-786 (1997).
9. Burt, A., Carter, D. A., Koenig, G. L., White, T. J. & Taylor, J. W. Molecular markers reveal cryptic sex in the human pathogen *Coccidioides immitis*. *PNAS* **93**, 770-773 (1996).
10. Fisher, M. C., Koenig, G. L., White, T. J. & Taylor, J. W. Primers for genotyping single nucleotide polymorphisms and microsatellites in the pathogenic fungus *Coccidioides immitis*. *Mol Ecol* (in press) (1999).
11. Brown, A. H. D., Feldman, M. W. & Nevo, E. Multilocus structure of natural populations of *Hordeum spontaneum*. *Genetics* **96**, 523-536 (1980).
12. Maynard-Smith, J., Smith, N. H., O'Rourke, M. & Spratt, B. G. How Clonal Are Bacteria? *PNAS* **90**, 4384-4388 (1993).
13. Archie, J. W. A randomization test for phylogenetic information in systematic data. *Syst. Zool.* **38** 239-252 (1989).
14. Crow, J. F. & Kimura, M. *An introduction to population genetics*. (Harper and Row, New York, 1970).
15. Swatek, F. E. & Omieczynski, D. T. *Coccidioidomycosis* (ed. Ajello, L.) (The University of Arizona Press, Tucson, Arizona, 1967).