

STEPHEN J. O BRIEN

GA: ... Our next speaker is Steve O'Brien. Steve O'Brien is truly a Renaissance biologist. As chief of the Laboratory of Genetic Diversity, one is as likely to see Steve announcing another major breakthrough in retrovirus and AIDS research just before heading to an IUCN field and specialist group meeting to discuss Amur leopard conservation. A little bit different than for many of us.

Of special note is Steve's founding of New Opportunities in Animal Health Sciences program at The Smithsonian Institution National Zoological Park. The conservation-genetics community has benefitted greatly from having Steve bring his energy, intellect and resources to this field. It's my great pleasure to introduce Dr. Stephen O'Brien.

(Applause)

SPECIES CONSERVATION AND THE NEW GENOMIC TECHNOLOGIES

STEPHEN J. O BRIEN, *Chief, Laboratory of Genomic Diversity,
National Cancer Institute*

SO: Thank you, George. And also thank you Howard, and Rob and Eleanor, for putting together the first conservation genetics meeting. I'm really excited to be here, and I'm looking forward very much to hearing a couple of days of the latest advances in this very young field.

Now, by way of introduction I would say that genetics, itself, is kind of an old field—it's been around for 150 years—and it's a huge field, with implications in every corner and scaffolding of modern life sciences. The full genome sequencing of the human and the mouse genomes are near completion, and other genomes will be on the way. So it won't be long when we will be seeing a new mammal sequenced every year.

Conservation of species, however, is a young idea. In the expanding frontiers, bush and wildlife were traditionally a challenge to be tamed, cleared, conquered and developed. The grazers and predators were extirpated deliberately and effectively, as generations of explorers, settlers, farmers, real-estate developers rendered the wildlands hospitable, habitable, for a massively growing human population.

It was really less than 50 years ago that the troubling consequences of such adventures and exploitations were recognized and pronounced. Authors like Rachel Carson, *Osa Johnson, Karen Blixton, Norman Myers, George Schaller trumpeted the irrefutable message that the earth's biodiversity were threatened with the most accelerated extinction process ever. And the cause was not a galactic meteorite, a global plague or a climactic catastrophe. The driver of the greatest extinction in the history of our planet was us.

Today, people of every nation recognize the imperative to reverse the depredation of threatened wildlife. And as I've traveled to the four corners of the earth, it is not hard to find champions on behalf of species conservation.

The field of environmental and species conservation spans a wide array of academic disciplines. These include ecology, politics, sociology, history, law, medicine, genetics, reproduction, behavior, physiology—and that's just a few of the subdisciplines of conservation.

My own field, molecular genetics—the emphasis of this conference—receives a lot of attention, a lot of journal ink, and for a good reason. It offers

enormous potential in virtually every field of biology, including species conservation. However, it should always be viewed as simply a part of the conservation story of any species.

In every situation its import and influence is very different. Yet I believe that the understanding of the genetic structure of threatened and endangered species informs us of the potential and perils that cannot be viewed by peering through binoculars, however carefully.

Conservation genetics as a field is less than two decades old. It began with the discovery of threatened populations whose number had dropped very low, and in some cases carried a genetic hangover from their narrow escape from extinction. Small free-ranging populations would increase the likelihood of close inbreeding. And if the low numbers persist, they would sometimes but not always or even predictably show correlates of inbreeding.

These correlates could include congenital abnormalities, reproductive impairments, or monotony of the immune response to infectious disease. Because microbial pathogens can rapidly evolve mechanisms to abrogate immune responses among individuals, genetically depauperate populations will increase their vulnerability to adapted microbes often as a prelude to the last act in an extinction tragedy.

We learned these lessons originally from the African cheetahs and the northern elephant seals. But over the years my colleagues and my friends have discovered additional genetically homogenized populations, such as the Gir Forest lions, the Florida panthers and several others. Yet, as you'll see from the diverse topics of this conference, conservation genetics has extended way beyond the estimates of heterozygosity and relative diversity.

This slide shows some of the subdisciplines which are relevant to conservation, in which molecular genetic assessment has been applied. Each of the applications shares a common thread: to reveal aspects of natural history of a population of species by interpreting the diversity patterning of different categories of genome families in an archeological sense. The challenge is to uncover genetic footprints of historic events, which molded the species to adapt, to develop, to survive and, in some cases, to stumble.

The goal is to equip the conservators of threatened species with real-time data and information, with which they can identify the perils of these threatened populations explicitly, and use that information to make management decisions which are based on solid data, as opposed to guesswork.

I'd like to illustrate these applications with a few examples that my students, postdocs and colleagues have had the privilege to touch during our work. The fields that conservation genetics involves and you'll hear about all of them at this conference as I said started out with diversity. But it's also been extended to fine-tuning taxonomy and systematics.

Phylogeography a word coined by John Avise, which really means taking phylogenetic analysis and seeing whether it can reveal population structure as a result of isolation over time. Behavioral ecology testing the hypotheses about mate selection and competition in the natural setting. Coalescent dating is a technique that is used to really look back how far back certain events that we can identify by their footprints in our genomes, or in the genomes of species how far back they took place. When it was that bottlenecks took place or migration events, or speciation.

Biomedical ecology is a field where pathogens, viruses and certain kinds of environmental microbes are tracked to emerge between different species, and are understood as threats not only to the animals, but also to humans. Gene mapping is, of course, a field that has enormous potential in conservation. Because we hope, in the future, to be able to actually start asking questions

about the genes involved in adaptation, explicitly, rather than simply using these anonymous markers that track the genomes. And forensics, of course, is applications for molecular genetics with respect to identifying endangered species that are illegally transported but it's also been used because, occasionally, individuals leave parts of animals at the scenes of crimes. These are just some of the things that population genetics gets into.

As I mentioned, our group started becoming interested in endangered species when we were introduced to the African cheetah and its problems. And the cheetah, of course, was remarkable, because it had a highly homogenized genome, with respect to other cats or other species. And when we first looked at this there were about 1,000 other species that had been looked at with molecular variation the cheetah looked like a deliberately inbred strain of laboratory mice.

We looked at a number of different markers which showed the story. And the reason that it was so genetically monotonous is that we believe it escaped a near-extinction event about 10,000 years ago, at the end of the Pleistocene, when it shared the planet with species like the sabre-toothed tigers, the American lions, the giant ground sloths. All of which went extinct at the end of the Pleistocene.

But before that period the cheetah had a range which included North America, Asia and Africa. Afterwards, it had escaped extinction, and pockets of cheetahs were found in Africa, and led to the population that exists there now. But it came out with a genetic hangover, which was a monotonous genome, which was between 95 and 99% reduced of the overall diversity that its ancestors and other cats show.

Since then, we've looked at a number of other species and discovered several that have had also genetic homogenization as the result of dropping to small numbers. One of the most interesting ones was the Florida panther. But this wasn't an ancient genetic homogenization it was one that took place as the result of simply clearing out the habitat in the southern United States over the last 150 years or so.

The Florida panther showed correlates of inbreeding, in the sense that it had morphological characters such as kinked tails at high frequency, or cowlicks. It also had congenital abnormalities, such as heart atrial septal defects, such as a hole between the two atria in the heart. It had reproductive problems the highest frequency of abnormal sperm of any cat we've ever looked at. The Florida panther has ninety-five percent of the spermatozoa in any ejaculate is morphologically malformed.

So, as a result of this, the conservators of the Florida panther made a decision several years ago to supplement the population with an introduction of pumas which the Florida panther is a subspecies of from a neighboring subspecies, the Texas cougar *puma concolor stanleyae*. And this decision was really made with the idea that it would simply reconstitute gene flow between two subspecies that *had* exchanged genes about 150 years ago.

After about four years of bureaucratic jostling over whether or not this was a good idea, the experiment was actually undertaken. And a half a dozen cougars from Texas were introduced into the Florida habitat, where a tiny population of about 30 animals was hugging to the edge of extinction with all these correlates of inbreeding and problems.

The result of that, which is still being analyzed, seems to, at least anecdotally, been a terrific success. There were five of the animals that were successful in producing offspring, and those animals were very vigorous. They ran so fast the dogs had trouble catching them. And, as Melody Roelke and Roy McBride explained to me, in the old days, when they treed a Florida panther, it

was sitting up in the tree and shivering, and weak, and really rather motley-looking.

But the new hybrid animals, between the two subspecies, are so strong that, when they do get treed, they simply jump out, over the dogs and the biologists, and run away. So you're talking about a number of at least anecdotal correlates and, we think, other things that have shown that one generation of introduction and abrogation, if you will of the genetic homogenization, has really been a step in the right direction in that particular situation.

Well ... most of you won't recognize this critter as an endangered species. What he is is an artist's rendition of the small insectivore, or rodent-type species, that was supposed to have been the prelude to all the mammals that exist today. It's 150 million years ago. It's reconstructed from a fossil that was uncovered in China a few years ago. And I put this up here because I think that, in order to understand and to save today's species, it's good to look back and understand a little bit about where we all came from.

Well, a couple years ago I was involved in trying to understand the evolution of mammalian radiations of which there are about 4,500 species by taking a look at the organization of the chromosomal arrays, by comparing gene maps of species for which there were gene maps. And, in the process, I called up a lot of the experts in the area of molecular evolution, and morphological evolution and paleontology, and attempted to come up with a consensus view of the hierarchy of the mammalian radiations. In other words, what is the phylogenetic relationship? And what I discovered is, there was no consensus but there was a lot of contentious topics.

Having discussed this with my postdocs and graduate students, they decided that they would like to take a shot at trying to sort this out. And I just want to tell you a little bit about what they found, because it's the kind of thing that grows out of these kinds of discussions.

As I said, the mammals consisted of about 4,800 species living today. They have been classified as placental mammals, and marsupials and monotremes. Among the placental mammals there are 18 recognized orders, which are listed right here. And so what Bill Murphy and Eduardo Eizirik did was, they rounded up through a lot of people in this room, as well as other scientists individuals from each of these orders multiple individuals and attempted to use the pattern of nuclear gene evolution to try to understand, or reconstruct, the relationship between these species.

The way they did it was to design PCR primers, which were universal for all mammals, by aligning sequences which are in the databases between human, mouse and artiodactyls usually cow. And then using those conserved primers on species from other orders, that we didn't know what the sequence was. And crossing your fingers, and hoping that they amplify and allow the sequence.

This worked a lot for very conserved genes. In fact, this slide here is simply a relative rate of evolution of about 250 genes that we were able to design primers for. And, as you can see, there's a tenfold difference in the rate of evolution of different genes. Some of them are going very fast this is the extent of difference between mammalian versions of the same genes and this is the slowest. So we selected about 15 genes that go very slow and said: Can we resolve the deepest divergence nodes of the mammalian radiations, at least? Or can we come up with a tree that makes any sense?

These are the 14 genes we looked out. All together, there was about 10,000 base pairs, and we were successful, really, in amplifying about 95% of the genes in each of the species. Not all of them worked. This paper came out about a month ago, in *Nature*, which simply shows what the data show. The data say that most of the orders that had been recognized by molecular and

morphological methods made sense. But, probably, the punch line was that the mammalian orders are really broken up into four major clades, or groupings, that are illustrated on this slide right here.

The first one is a group that the molecular evolutionists discovered a few years ago called Afrotheria. It's a series of orders including elephants; and sirenians, or the manatees; the tubulidentates, or armadillos; afroceridia, core insectivores; and a few other species that have their origins in Africa.

The second which was highly supported by, at least, the molecular data was the South American Xenarthra the sloths, anteaters and armadillos. The third group consists of the primates, and the rodents, and the relatives of the primates the tree shrews and the lemur forms. And then the fourth was the rest of the species. A better, more visible, version of this tree is shown in the next slide, so I'll move to that...

And, basically, again, there were four clades: the Afrotheria, which is this group which were aligned with a degree of statistical confidence, at least for these genes the Xenarthra, the South American; then the primates. And the traditionally aligned, morphologically The morphologists seem to like the grouping of the lagomorphs, the rabbits and the rodents we were able to affirm that quite strongly with our data and then the grouping of the artiodactyls, the parasitactyls; the whales, the pangolins is a sister taxon with this group; and then the bats and another insectivore group.

We did affirm the polyphylae of the insectivores pretty strongly. And when we attempted to date the nodes of the four major groups, they all came out prior to the KT extinction estimated at between something like 80 to 100 million years ago. And when we look at the geological record, it actually makes a little bit of sense. Because 100 million years ago, the southern continents were one big huge Gondwanaland continent.

And then, subsequently, about the time of these divergence nodes, they broke into Africa and South America. And, of course, this is, basically, the physical position of the Afrotheria species and the Xenarthra species. And then, we believe, after that, there was a migration north that led to the radiations of the others. That's the abstract of what this data showed. There's lots of other implications, but the tools of molecular genetics are getting pretty good.

We tackled another question, which was more local, about the subspecies of African elephants. There's two subspecies that have been used, and we use the same kind of method that is, nuclear markers which were with the universal primer the comparative anchor tag sequence, we call them CATS primers to simply see if there was a difference between the ecologically diverse forest element and the savannah, or bush, element.

And, basically, these are a little bit smaller. And what happened is, we collaborated with Nick Georgiades, who spent about six years traveling throughout Africa with a dart-biopsy gun and collected over 300 elephants from throughout the range. And three of the populations, located right here, were classified by him and his colleagues as being forest elephants, and the rest of them were all savannah elephants.

And there have been some descriptions that indicate that there are morphological differences particularly in size and in the shape of the tusk between these elephants. But the universal opinion about these elephants is that they're a different subspecies.

Well, the genes didn't quite agree. When we analyzed those populations, what we discovered was that when we looked at the savannah elephants, using the nuclear genes, we found a rather monotonous set of virtually identical sequences. Over 3,000 base pairs, which looked as if there'd been a founder effect in the history of the savannah. And you remember the savannah was

the one ranging throughout Africa. But in the forest elephant these three tiny populations there a *rich* amount of diversity here.

But the interesting part was that there was a major difference between them. And this outgroup is the Asian elephant, and the genetic distance between the savannah elephants and the forest elephants turned out to be of the order of about 3 million years or about three-fifths of the distance between the genera of Asian elephants and African elephants. So what has traditionally been called a single species had genetic distances which were comparable to the distances between lions and tigers, or even between chimpanzees and humans.

This led us to make the proposition that we might want to rethink the taxonomy of the species, and perhaps increase the number of species in proboscidea order from two to three. This is simply one of the subdivisions of the genes. It was an X-chromosome gene with about 15 polymorphisms, or single nucleotide polymorphisms. We were able to haplotype these. Because they were on the X, we could tell the haplotype in males. And it just simply shows that the Asian elephants had this level of steps from the savannah vs. the forest, and it illustrates that the diversity is quite homogeneous, or quite distinct, between the two.

Furthermore, even though there was regions of physical overlap, our data was able to detect heterozygotes. And we detected none, out of 300, between the African and the savannah elephants. There seems to be a reinforcement of genetic isolation between these species.

We use similar kinds of methods on the cat family, of which there s 36 species all together. In addition to the house cat, trying to solve a 12-million-year-old radiation. We still haven t settled it completely, but we do have evidence for using these different methods. Over the last 10 or 15 years we ve had the luxury of being able to use all of the tools of molecular evolution on the cat family.

Basically, what we think is going on and we have evidence for from multiple methods is eight groups of cats. With the basal one being the ocelot lineage, which consists of seven species of cats related to the ocelot. The next group is the house-cat group, or the small genus *Felis*, and then there s a group with the puma and the cheetah; there s a group with the great cats; there s a group with the lynxes, Asian leopard cat group, and so forth. And, probably, we ll eventually come out and recommend that the generic names be changed to reflect the timing of divergence, and the monophylae that we re seeing with these particular methods in the cats.

Finally, we ve looked at subspecies partitioning in the cats, as we have in the African elephants, using First of all, we used last year s methodology allozymes, mitochondrial DNA and minisatellites, using a collection of leopards from zoos and wildlife parks throughout the world and we were able to come up with a graduate student, Sriyanie Miththapala, to reduce the number of subspecies from 30 down to about eight. And they re indicated by these different colors here.

The next thing we did is, we extended that, using DNA sequencing of mitochondrial genes as well as composite microsatellite markers, which are powerful, small, repeat sequences in the genome that are useful for tracking recent events. The mitochondrial analysis of the leopards indicated a good phylogeographic partitioning between the groups that I indicated a few minutes ago where most of the African populations were polyphyletic; they had a big grouping.

And, yet, there was a partition between Asian, Indian, Sri Lankan and East Asian. When we looked at microsatellites, we reinforced the same idea that is, these are individuals from *fusca*, which is India; from *kotiya*, which is Sri Lanka; *delacouri*, which is Southeast Asia; *japonensis*, which is Chinese and

Japanese; *orientalis* is the Russian Far East the north Amur leopards, and the outgroup is tigers.

So, basically, the microsatellites and the mitochondrial DNA both gave us the same kind of partitioning and association which gave us a more robust, objective criterion for recognizing subspecies. Something which both John Avise and I have recommended for the recognition, particularly for the legal aspects of it.

But, interestingly enough, the amount of diversity in these places is a little bit different. As I said, the major amount of diversity is found in Africa, and the base of the tree was found in the *pardus* subspecies, which is from Africa. The dating of this particular diversity goes back to about 400,000 years for the *pardus* itself. But, out of Africa, the diversity up here is less so as if there was a migration out of Africa, as for humans. And the time of that goes back to about 150 to 200,000 years ago. Which is *exactly* the same time that humans migrated out of Africa. So it looks like when the humans migrated out, they took the leopards with them. Don't ask me why. (Laughs)

We did the same kind of thing for the pumas. We had a graduate student, Melanie Culver, who went throughout the range of pumas and collected over 300 individuals partitioned the pumas into six major subspecies, and, with microsatellites, discovered something rather interesting. Which is, these are simply the distribution of allele sizes for certain loci, with South American pumas versus North American pumas.

And what was discovered is that there's a wide breadth of diversity in the North American, but it's narrow in the South American that is, there are only a few alleles. That indicates that there probably has been a recent founder effect in North America which we were able to actually date using the breadth of diversity, and calibrating it using the African cheetah's dating and the divergence.

And when we did that, what we discovered was that the puma had a founder effect in North America about 10,000 years ago. That didn't make a lot of sense, because there's a lot of fossils in North America from pumas. So what we think happened is that whatever knocked out most of the large mammals in North America 10,000 years ago, at the same time the cheetah disappeared, the sabre-toothed tigers, the giant ground sloths, the things that are upstairs all those early mammals that are upstairs in this museum when they got knocked out, we think the pumas got knocked out, also, and then were repopulated by a small number from South America.

Those are the kinds of questions we can ask and answer. You'll hear more at this meeting. I didn't do any of this work. It was done all by my friends and my students. Naoya Yuhki, Bill Murphy and Eduardo did the mammal evolution; Melody Roelke was a major player in the puma story; Melanie Culver, her graduate thesis was the puma coalescence. These are some of the people in the laboratory at our retreat that were working on all this. And I just want to say that it really is the technology of human genetics that has allowed us to ask some of these questions in these animals, and that we're really excited, now, to hear some more from the rest of this group.

Thank you very much.

(Applause)