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The Effects of Ionizing Radiation on Domestic Dogs: Ancestry and Genetic Structure of Free-Roaming Dog Populations in Chernobyl, Ukraine

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The Effects of Ionizing Radiation on Domestic Dogs: Ancestry and Genetic Structure of Free-Roaming Dog Populations in Chernobyl, Ukraine

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Dedication

To my mom and dad; thank you for always supporting me and encouraging me to pursue my passions.

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Abstract

This thesis is concerned with the effects of ionizing radiation on domestic dogs (*Canis lupus familiaris*) which were commonly used as laboratory subjects during the era of atomic bomb testing (1950s - 1980s). Early laboratory studies on radiation effects in dogs provided important foundations of knowledge for research that has more recently shifted to natural populations exposed to ionizing radiation from nuclear accidents. A unique group of free-roaming dogs reside in an environment polluted by radioactive contamination deposited during the Chernobyl nuclear disaster of 1986, providing one such opportunity to study the effects of ionizing radiation on dogs in a natural setting. In addition, these populations offer opportunities to examine the evolution of dog populations following 30+ generations of free-breeding. The current free-roaming dogs in Chernobyl are thought to be descendants of pets left behind during the evacuation of cities and towns following the nuclear disaster. This study implemented analyses using SNP genotyping to investigate the population structure and genetic diversity of these canines in an attempt to better understand their ancestry and origin in the area. The Chernobyl dog population was highly genetically varied compared to village dog populations from neighboring countries. Further testing is required to investigate the true cause of increased genetic variation found within the Chernobyl population, as it could potentially relate to radiation exposure.

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Chapter 1

The Effects of Ionizing Radiation on Domestic Dogs: A Review of the Atomic Bomb Testing Era Literature[1]

Abstract

Chapter one of this thesis is a literature review of background knowledge on the effects of ionizing radiation exposure on domestic canines for use in future studies. Such a review is novel and necessary to generate predictions concerning expected effects of Chernobyl-derived contaminants on dogs in the area. Although dogs were frequently employed as laboratory subjects during the era of atomic bomb testing (1950-1980), many studies at the time rarely made it to the primary literature. Here, I summarize and tabulate the bulk of these studies, most of which were partly reported in the primary literature; however, many were extracted from government documents and reports not easily available to the general public. Overall, these studies demonstrate the wide range of developmental and physiological effects of exposure to radiation and radionuclides stemming from the products of nuclear fission.

[1] Spatola, G.J. and Mousseau, T.A. To be submitted for further publication.

Introduction

Studies on the effects of ionizing radiation exposure in animals were largely initiated in order to determine safety guidelines for humans working with radiation and remain of interest to biologists, even today. The insight gained from studies of radiation exposure can be used to better prepare for future nuclear accidents where humans and other species may be unintentionally exposed.

Domestic dogs were a prominent study organism for early radiation studies. Large scale, long term studies investigating the effects of radiation exposure on domestic dogs first began in the early 1950s and were originally supported by the United States Atomic Energy Commission (AEC), which was established after the end of World War II to advance and control atomic research and technology. The AEC was disbanded in 1974 and its functions were reassigned to the Energy Research and Development Administration, later known as the U.S. Department of Energy. These agencies supported several large-scale studies designed to test the effects of radiation exposure on the health and lifespan of dogs, particularly beagles.

Radium and plutonium were the first internally deposited radionuclides involved in biological studies of long-term effects on dogs (Stannard 1988; Thompson 1989). Radium isotopes were studied in response to the discovery of exposure effects in people working in dial-painting plants. These workers would “point” their brushes using their tongues causing them to ingest small amounts of radium in the paint. Interest in plutonium stemmed from its use in atomic bombs where guidelines for plant workers were previously based on radium levels

derived from limited research on people accidentally exposed to the radium paint (Thompson 1989; Dougherty et al. 1962). Research using domestic dogs to study radiation exposure effects expanded as interest in nuclear power sources gained traction, resulting in more fission product radionuclides being added to large scale studies. Fission product radionuclides are components of fallout from nuclear weapons testing, nuclear reactors, and nuclear-powered rockets, all of which were new technologies that heightened apprehension for accidental exposures (Thompson 1989).

Domestic dogs as a species were chosen for radiation exposure research because of their size, lifespan, and availability, among other factors. Early on, stray dogs were often used for research purposes, however, as technology progressed researchers gained a better understanding of confounding health history and genetic factors, which were unknown in strays, causing studies to shift in focus to purebred dogs. Beagles were selected largely for their non-aggressive nature and availability (Thompson 1989; Andersen and Good 1970). Two radiation exposure studies tested effects on Saint Bernards rather than beagles, for the purpose of comparing resulting effects to those found in beagles using similar experimental methods (Taylor et al. 1981; Lloyd et al. 1983c). Saint Bernards were chosen in these studies because they are naturally susceptible to bone diseases, providing researchers with the opportunity to better understand how natural susceptibility influences radiation-induced bone disease.

Early studies of radiation exposure effects on dogs were done using very limited sample sizes and set the foundations for similar but larger scale studies

that followed. For example, early studies done at the University of Rochester in New York involved inhalation of polonium, uranium or plutonium oxides, or radon daughters, however these studies were short term, involved limited sample sizes, and rarely made it to peer reviewed scientific literature (Thompson 1989). In June of 1960, the Lovelace Foundation for Medical Education and Research in New Mexico was provided with funding for the foundation of a new laboratory with the ability to construct and operate larger scale inhalation studies on dogs. This new laboratory, later known as the Inhalation Toxicology Research Institute (ITRI), was largely staffed by Rochester graduates (Thompson 1989), and the studies completed by this laboratory provided more convincing evidence of radiation exposure effects.

Many of the large-scale studies were followed by smaller ancillary studies, yielding an overall broader scope. For example, major studies tended to include young adult dogs, but several smaller scale studies of juvenile or aged dogs were done in order to investigate the influence of age or development on differences in radiation exposure effects (i.e. Lloyd et al. 1983a; Lloyd et al. 1983b). Similarly, some ancillary studies were done with minor variations in exposure methods, such as altering the particle size of inhalants or using radionuclide aerosols of differing solubilities, to determine if these alterations would cause substantial differences in exposure effects (i.e. Guilmette et al. 1984; Muggenburg et al. 1996). We discuss these ancillary studies in comparison to their respective major studies in order to provide the most comprehensive and logical understanding of radiation exposure effects on domestic dogs.

The effects of internally deposited radionuclides depend on their distribution, retention, and length of duration within the body. The initial absorption of radionuclides differs based on their entry into the body. Studies on domestic dogs implemented four primary methods of radiation exposure, including intravenous injection, inhalation, ingestion, and external irradiation. For this reason, we grouped studies based on the method of radiation exposure.

Discussion

Intravenous injection

Intravenous injection was chosen as a primary method of exposure because it was thought to bypass the complications of absorption (Thompson 1989). Twenty-one major studies exposed dogs to intravenously injected ^{137}Cs, ^{144}Ce, ^{226}Ra, ^{224}Ra, ^{228}Ra, ^{228}Th, ^{239}Pu, ^{241}Am, ^{249}Cf, ^{252}Cf, ^{253}Es, or ^{90}Sr (Tables 1.1, 1.2, 1.3, and 1.4). The most prominent effects of intravenously injected radionuclides were bone and skeletal tumors, which were subsequently leading causes of death (White et al. 1994; Lloyd et al. 1993; Lloyd et al. 1995; Bruenger et al. 1980; Lloyd et al. 1994). Radioactive elements such as strontium, radium, and plutonium are notoriously bone-seeking, so elevated retention in these parts of the body after injection leads to much higher effective doses to these tissues. A similar trend was seen with I^{131} released after the Chernobyl nuclear disaster. Iodine is naturally concentrated in the thyroid, so children exposed after the nuclear disaster received higher doses to the thyroid compared to the average body dose, resulting in increased incidences of thyroid cancers.

Further effects of intravenously injected radionuclides included liver tumors and hematopoietic cell damage. Beagles intravenously injected with ^{137}Cs and ^{241}Am solutions had increased incidences of liver tumors (Nikula et al. 1995; Lloyd et al. 1995; Nikula et al. 1996). At Argonne National Laboratory, all middle-aged dogs exposed to ^{137}Cs died from complications associated with radiation induced hematopoietic cell damage (Nikula et al. 1996).

Hematopoietic cell damage is now known to be a leading cause of death after exposure to ionizing radiation and was first reported in a few dogs exposed to large doses of X-ray emissions in 1922 (Shao et al. 2014). Damage to hematopoietic stem cells via ionizing radiation causes differentiation and suppression of bone marrow (BM) development and is dependent on the radiation dose (Shao et al. 2014; Guo et al. 2015). Bone marrow (BM) suppression and hematopoietic cell damage are well known direct results of oxidative stress, although the related mechanisms are debated (Shao et al. 2014). As a result of the BM damage, exposed subjects may develop aplastic anemia or a myeloproliferative disorder, where bone marrow cannot correctly produce blood cells. This often initiates a cascade of effects that ends in mortality.

An additional effect of intravenous exposure to ^{137}Cs included testicular damage in male beagles. All long-term male survivors injected with ^{137}Cs were aspermatic (Nikula et al. 1996). More recently, reported findings of testicular damage by ionizing radiation exposure were supported in an experiment using a ^{137}Cs source to expose several groups of male mice to gamma radiation at

various levels (Son et al. 2015). This study indicates that radiation, even at low dose rates, can be used to induce disruption of the blood-testis barrier (Son et al. 2015). The blood-testis barrier helps to protect the germ cells and maintain the appropriate microenvironment; however, disruption causes infertility in the male mice. Evidence of infertility due to radiation exposure was also reported in wild caught birds from Chernobyl, Ukraine, where ^{137}Cs was among one of the most concerning radionuclides apparent after the nuclear disaster (Møller et al. 2014). The study by Møller et al. (2014) investigates aspermy and sperm quality in birds that were externally exposed to relatively low doses of radiation consistently via the environment in and around Chernobyl. The research shows that the rates of male birds with aspermy increased logarithmically with the level of radiation exposure, and 18.4% of males from highly contaminated areas were absent of sperm altogether. Studies on mammal populations environmentally exposed to ionizing radiation are needed to further understand these findings.

Retention of intravenously injected radionuclides differed among dogs of different age classes. Juvenile dogs injected with either ^{226}Ra or ^{239}Pu solutions appeared to have greater retention in the skeleton compared to young adults similarly injected (Lloyd et al. 1983b; Bruenger et al. 1980). Retention of ^{226}Ra or ^{239}Pu was also higher in young adults compared to aged beagles (Lloyd et al. 1983a). Nikula et al. (1996) analyzed data from two laboratories testing the effects and survival of beagles injected with ^{137}Cs at different ages. This study found that aged dogs of both genders died significantly earlier than juvenile or young adult dogs, suggesting that radiosensitivity is age dependent.

Only two early radiation studies examined effects in Saint Bernards rather than beagles, and both of these studies exposed Saint Bernards intravenously. Saint Bernards exposed to intravenous ^{239}Pu appeared to be more susceptible to radiation induced bone tumors than beagles similarly injected, which was expected given their natural predisposition to bone cancer (Taylor et al. 1981). In addition, Saint Bernards retained more intravenously injected ^{226}Ra than beagles, potentially contributing to their increased susceptibility (Lloyd et al. 1983c).

Inhalation

The most likely route of human occupational exposure to radionuclides was thought to be inhalation, leading researchers to implement this mode of exposure in animal models (Thompson et al. 1989). In 25 major studies, dogs were exposed to ^{239}Pu, ^{238}Pu, ^{144}Ce, ^{90}Sr, ^{90}Y, ^{91}Y, or ^{241}Am by inhaled aerosols containing radionuclides (Tables 1.5, 1.6, 1.7, 1.8, 1.9, 1.10, and 1.11). Lung tumors and respiratory damage were common effects following inhalation of radionuclide aerosols (Hahn et al. 1997; Clarke and Bair 1964; Muggenburg et al. 1996; Bair and Willard 1962; Park et al. 2012) and were unique to this method of exposure. Radiation pneumonitis, which is inflammation of the lung caused by radiation exposure, was the predominant non-neoplastic disease found in dogs exposed via inhalation (Hahn et al. 2001; Hahn et al. 1997; Muggenburg et al. 1996; Hahn et al. 1975). Radiation pneumonitis is now known to be a common effect in human lung cancer patients who receive chemoradiation treatments. Radiation pneumonitis is often not fatal in human patients, although it is

associated with high daily radiation doses and lower-lobe lung tumors (Palma et al. 2013).

After brief retention in the lungs of dogs, some radionuclides tended to translocate throughout the body, causing differences in effects related to deposition and protracted exposure. For example, a year after exposure to ^{238}Pu, retention was observed in the liver and skeleton of dogs where it persisted beyond 1,000 days after exposure; however, ^{239}Pu remained in the lungs of exposed individuals (Muggenburg et al. 1996). The leading cause of death reported in two separate studies of dogs exposed to ^{238}Pu aerosols were bone tumors, followed by lung and liver tumors, all of which appeared approximately three years post-exposure (Muggenburg et al. 1996; Park et al. 1997). ^{144}Ce similarly translocated to the liver, bone and bone marrow of exposed dogs, where subsequent occurrences of neoplasms were elevated (Hahn et al. 1997). Long-term retention of inhaled ^{90}Sr was highest in the bone tissues of exposed dogs, leading to protracted exposure of the skeleton (Gillett et al. 1987b; Benjamin et al. 1975). Translocation of radionuclides after exposure likely influences delayed effects, where the deposition of radionuclides causes protracted exposure and an overall higher dose to these organs and tissues well after the initial exposure.

Ingestion

Only one major study used ingestion as an experimental method of radiation exposure despite this being a primary route of longer-term exposure after nuclear accidents (Table 1.15). After the explosion of a nuclear power source, radioactive fallout settles and people are likely to be exposed by

ingesting contaminated foods. This experiment was intended to investigate the effects of indirect exposure to unborn children who are exposed by their pregnant mothers living in areas where nuclear fallout has settled. Pregnant dams were fed ^{90}Sr at various doses and pups were continued on the diet until 540 days after birth. In the highest dose exposure groups, major effects included bone tumors, myeloproliferative disorders, and shortened lifespans (White et al. 1993; Book et al. 1982; Dungworth et al. 1969). The distribution of bone sarcomas that the dogs developed was correlated with the cancellous bone volume-to-surface ratio rather than bone mass or dose distribution (White et al. 1993). Interestingly, beagles that ingested the lowest dose (1.3 μCi/day) appeared to have normal lifespans and did not develop any radiation induced bone disease (Book et al. 1982).

The effects of exposure to low dose ionizing radiation remain of interest to biologists, even today, as these effects are often long delayed or confounded by other environmental factors (Burlakova et al. 2016). As radiation dose decreases, the uncertainty to which these effects can be directly attributed to radiation increases. This uncertainty makes it necessary for researchers to use very large sample sizes, which is often not possible in studies on larger mammals, such as dogs, that are expensive and labor intensive to keep.

External X and gamma ray exposure

External exposures were given to dogs using ^{60}Co gamma rays or X-rays in several studies commissioned by the AEC and in studies completed by the University of Ulm, Germany (Tables 1.12, 1.13, and 1.14). Unlike research using

internally deposited radionuclides, studies using external exposure methods were largely interested in studying leukemogenic processes, including hematopoietic function and characteristics of bone marrow after exposure. In dogs continuously exposed to ^{60}Co gamma rays, early hematopoietic failure was positively associated with accumulated dose and dose rate of exposure (Carnes and Fritz 1993). In dogs exposed in-utero or continuously in-utero through after birth, hematopoietic function was progressively suppressed until 100-150 days of age at which function was partially recovered, however neither group showed evidence of linked radioresistance by hematopoietic progenitor cells (Seed et al. 1987). An extensive large-scale study conducted at the University of Colorado, which exposed over 1500 dogs to ^{60}Co gamma rays in-utero to 12 months of age. This study found that fetally and neonatally exposed dogs were at an elevated risk for cancer (Benjamin et al. 1998b). Mortality related to neoplasia occurred in 40% of dogs exposed, with significantly increased occurrences in dogs less than four years old. All instances of myeloproliferative disorders or leukemias in this study occurred in dogs exposed in-utero or as neonates; however, these maladies did not appear until adulthood (Benjamin et al. 1998b). Hematopoietic responses after radiation exposure in dogs are now well documented and the mechanism for development of myeloproliferative disorders, as well as their progression into acute myeloid leukemia, have been studied extensively (Seed et al. 1993; Seed et al. 2002; Seed and Kaspar 1985; Seed et al. 1988).

Hematopoietic responses have also been documented after accidental exposure to ionizing radiation in humans. Kesminiene et al. (2008) investigated the risk for hematological malignancies in clean-up workers (liquidators) exposed after the Chernobyl nuclear disaster. Liquidators appeared to be at a significantly increased risk for hematological malignancies at overall doses of 200 mGy or above, although several potential issues with the dose reconstructions were presented. 117 neoplasms of lymphoid and hematopoietic tissue were reported out of 481 possible controls, 69 of which were diagnosed as leukemia. The association between accidental exposures and resulting instances of leukemia are debated, especially at low dose exposures.

Other methods of exposure

In addition to the primary exposure methods mentioned above, three studies exposed dogs via subcutaneous injection, subcutaneous implants, and transplacental injection (Table 1.15). No results have been published in the primary literature regarding the studies that implemented subcutaneous injection and transplacental injection, however a summary of early results may be found in the International Radiobiology Archives of Long-Term Animal Studies (Gerber et al. 1996). Dogs injected subcutaneously with ^{90}Sr had higher instances of bone tumors and occurrences of myeloid leukemia were also reported (Gerber et al. 1996). Injections of two litters were terminated due to excessive mortality. In pups that were transplacentally injected with ^{90}Sr, bone tumors occurred at higher doses.

At the University of Colorado, dogs were implanted with ^{239}Pu subcutaneously in their forepaws to imitate hand wounds received by accidentally exposed workers (Dagle et al. 1984). Highest retention occurred in the lymph nodes of dogs, followed by the liver. Researchers concluded that there are no unique risks associated with this method of exposure as opposed to other major exposure methods (Dagle et al. 1984).

Conclusions

With an increasing demand for nuclear power comes higher risk of nuclear accidents. Studies on radiation exposure effects in domestic dogs provide valuable knowledge that is immediately applicable to populations who are accidently exposed. For example, particulate plutonium was detected in fallout released from the Fukushima Daiichi Nuclear Power Plant after the accident in 2011, although the physical and chemical form was unknown until recently. Kurihara et al. (2020) reported the discovery of plutonium associated with cesium rich microparticles (CsMPs), which are consequently released after nuclear accidents. When nuclear meltdowns occur, CsMPs are formed as a result of nuclear fuel reacting with concrete from the reactor's structure (Furuki et al. 2017). The discovery of plutonium released as a result of nuclear accidents highlights the utility of studies on the effects of plutonium exposure in dogs. Although experiments done in a laboratory setting provide valuable information, more studies are needed on natural populations affected by past radiological disasters in order to better understand how laboratory results may apply, as

these populations are affected by other potentially confounding environmental factors.

Domestic canines commonly share the same environment, lifestyle, and related pollutants as their human counterparts (Mazzatena 2017; Karlsson and Lindblad-Toh 2008; Switonski et al. 2004). Coupled with abundant genetic similarities to humans, this makes the canine model a useful tool in studying radiation induced diseases, especially in a natural setting. Hundreds of dog populations exist alongside residential areas across the globe, some of which live in areas contaminated by radioactive fallout from nuclear disasters or atomic bomb testing, such as in Chernobyl, Ukraine, Fukushima, Japan, the Semipalatinsk Test Site, Kazakhstan, or the Marshall Islands. These populations of dogs experience similar radioactive pressures as people living in the areas, so studying them may provide additional insights more relevant to human populations. Investigating the effects of radiation exposure on dog populations living in these areas is the next step in expanding our knowledge of the impacts nuclear accidents can have.

Table 1.1 Studies that exposed dogs to radiation via intravenous injection.

Radionuclide	Mode of exposure	Dose/Treatment range	Number of exposures	Age of dogs at first exposure (months)	Reference	Major results
137Cs	intravenous injection	9.6 - 14.6 Gy (cumulative dose)	single	5 - 68	Nikula et al. 1996	Liver degeneration; Aspermy; Hematopoietic failure
144Ce	intravenous injection	0.851 - 19.61 MBq/kg (total injected)	single	13	Summary of results available in Thompson 1989	Shortened lifespan; Bone tumors
226Ra	intravenous injection	0.789 - 370 kBq (total injected)	multiple	14.3	White et al. 1994; Raabe and Parks 1993; Raabe et al. 1981; Momeni 1978	Bone tumors
90Sr	intravenous injection	137 - 1220 kBq/kg (total injected)	single	17.7	Nilsson and Book 1987; Raabe and Parks 1993; Momeni et al. 1976	Bone tumors; Mouth tumors

Table 1.2 Studies that exposed dogs to radiation via intravenous injection (continued).

Radionuclide	Mode of exposure	Dose/Treatment range	Number of exposures	Age of dogs at first exposure (months)	Reference	Major results
224Ra	intravenous injection	13 - 380 kBq/kg (quantity injected)	single	15 - 24	Lloyd et al. 1982; Muggenburg et al. 1996	Bone tumors; Nasal tumors; Hematologic changes
226Ra	intravenous injection	0.74 - 37 kBq/kg (quantity injected)	single	3 - 5	Lloyd et al. 1983b; Bruenger et al. 1991; Lloyd et al. 2001b	Greater retention in juveniles
226Ra	intravenous injection	37 - 370 kBq/kg (quantity injected)	single	58.8 - 74.4	Lloyd et al. 1983a; Bruenger et al. 1991; Lloyd et al. 2001b	Lower retention in aged dogs; Kidney deterioration
226Ra	intravenous injection	0.02 - 1.1 µCi/kg (quantity injected)	single	17 (one was 110 months)	Lloyd et al. 1983c	Greater retention in Saint Bernards
228Ra	intravenous injection	0.74 - 333 kBq/kg (quantity injected)	single	13 - 24	Lloyd et al. 2001a; Dougherty et al. 1971	Bone tumors; Intraocular melanomas; Hematologic changes

Table 1.3 Studies that exposed dogs to radiation via intravenous injection (continued).

Radionuclide	Mode of exposure	Dose/Treatment range	Number of exposures	Age of dogs at first exposure (months)	Reference	Major results
228Th	intravenous injection	0.074 - 99.9 kBq/kg (quantity injected)	single	10 - 24	Lloyd et al. 1984a; Lloyd et al. 2001a; Dougherty et al. 1971; Stover et al. 1960	Bone tumors; Intraocular melanomas
239Pu	intravenous injection	0.037 - 111 kBq/kg (quantity injected)	single	13 - 25	Wronski et al. 1980; Lloyd et al. 1993; Peterson et al. 1982; Lloyd et al. 1999; Lloyd et al. 2001a; Dougherty et al. 1971; Bruenger et al. 1991; Lloyd et al. 2001; Cochran et al. 1962	Shortened lifespan; Bone tumors; Hematologic changes; Liver tumors
239Pu	intravenous injection	0.185 - 111 kBq/kg (quantity injected)	single	2.9 - 3.5	Bruenger et al. 1980; Lloyd et al. 1999; Bruenger et al. 1991; Lloyd et al. 2001b	Greater retention in the bone of juveniles; Bone tumors

Table 1.4 Studies that exposed dogs to radiation via intravenous injection (continued).

Radionuclide	Mode of exposure	Dose/Treatment range	Number of exposures	Age of dogs at first exposure (months)	Reference	Major results
239Pu	intravenous injection	0.592 - 11.1 kBq/kg (quantity injected)	single	49.2 - 62.4	Lloyd et al. 1978; Lloyd et al. 1999; Bruenger et al. 1991; Lloyd et al. 2001b	Greater retention on bone surfaces of aged dogs; Bone tumors
239Pu	intravenous injection	0.0158 - 0.903 µCi/kg (quantity injected)	single	19	Taylor et al. 1981	Bone tumors
241Am	intravenous injection	0.074 - 103.6 kBq/kg (quantity injected)	single	15 - 19	Lloyd et al. 1995; Lloyd et al. 1984b; Lloyd et al. 1994; Lloyd et al. 1970; Taylor et al. 1993; Polig and Jee 1984; Lloyd et al. 2001a; Polig et al. 1984	Bone tumors; Liver tumors; Thyroid damage; Hematologic changes; Liver failure; Kidney failure
249Cf	intravenous injection	0.0222 - 11.1 kBq/kg (quantity injected)	single	15 - 19	Bruenger et al. 1972; Lloyd et al. 1976; Lloyd et al. 1972	Bone tumors

Table 1.5 Studies that exposed dogs to radiation via inhalation.

Radionuclide	Mode of exposure	Dose/Treatment range	Number of exposures	Age of dogs at first exposure (months)	Reference	Major results
241Am	inhalation	180 - 500 rad (skeletal dose)	single	15 - 40	Mewhinney et al. 1982; Gillett et al. 1985	Translocation of radionuclides
239Pu	inhalation	0.4 - 14,000 rad (estimated total dose to lungs)	single	10 - 33.	Bair and Willard 1962; West and Bair 1964; Clarke and Bair 1964; Bair and Willard 1963	Translocation of radionuclides; Respiratory distress
239Pu	inhalation, 0.75-μm particles	0.518 - 5.92 kBq/kg (initial lung burden)	single	12 - 15	Guilmette et al. 1984; Hahn et al. 1999	Radiation pneumontitis; Lung tumors

Table 1.6 Studies that exposed dogs to radiation via inhalation (continued).

Radionuclide	Mode of exposure	Dose/Treatment range	Number of exposures	Age of dogs at first exposure (months)	Reference	Major results
239Pu	inhalation, 0.75-µm particles	0.703 - 11.1 kBq/kg (total mean deposition from exposures)	multiple	12 - 15	Summary of results available in Thompson 1989 and Gerber et al. 1996	Retention independent of exposure history
238Pu	inhalation, 1.5-µm particles	11.1 Gy (two-year mean dose to lung)	single	12 - 15	Muggenburg et al. 1996	Bone tumors; Lung tumors; Radiation pneumontitis
239Pu	inhalation, 1.5-µm particles	10.9 Gy (two-year mean dose to lung)	single	12 - 15	Guilmette et al. 1984; Hahn et al. 1999	Radiation pneumontitis; Lung tumors
239Pu	inhalation, 1.5-µm particles	0.0148 - 20.35 kBq/kg (initial burden)	single	2.6 - 3.6	Summary of results available in Thompson 1989 and Gerber et al. 1996	Lower incidence of radiation pneumontitis

Table 1.7 Studies that exposed dogs to radiation via inhalation (continued).

Radionuclide	Mode of exposure	Dose/Treatment range	Number of exposures	Age of dogs at first exposure (months)	Reference	Major results
239Pu	inhalation, 1.5-µm particles	1.11 - 13.69 kBq/kg (initial burden)	single	84 - 120	Summary of results available in Thompson 1989 and Gerber et al. 1996	Higher incidence of radiation pneumontitis
238Pu	inhalation, 3.0-µm particles	0.47 - 25 kBq/kg (initial lung burden)	single	12 - 14	Muggenburg et al. 1996	Bone tumors; Lung tumors; Radiation pneumontitis
239Pu	inhalation, 3.0-µm particles	12.2 Gy (two-year mean dose to lung)	single	12 - 15	Guilmette et al. 1984; Hahn et al. 1999	Radiation pneumontitis; Lung tumors
144Ce	inhalation, insoluble	0.333 - 3,774 kBq/kg (initial burden)	single	3	Summary of results available in Thompson 1989 and Gerber et al. 1996	Greater deposition in the skeleton of juveniles

Table 1.8 Studies that exposed dogs to radiation via inhalation (continued).

Radionuclide	Mode of exposure	Dose/Treatment range	Number of exposures	Age of dogs at first exposure (months)	Reference	Major results
144Ce	inhalation, insoluble	0.2923 - 1.998 MBq/kg (initial burden)	single	96 - 120	Summary of results available in Thompson 1989 and Gerber et al. 1996	Less deposition in the skeleton of aged dogs; Lung tumors
144Ce	inhalation, insoluble	92.5 - 333 kBq/kg (initial burden)	multiple	14 - 17	Summary of results available in Thompson 1989 and Gerber et al. 1996	Delayed lung tumors
90Sr	inhalation, insoluble (fused clay)	8.88 - 2,738 kBq/kg (initial burden)	single	11 - 15	Benjamin et al. 1975	Radiation pneumontitis; Respiratory tract tumors; Heart tumors
90Y	inhalation, insoluble	3.885 - 118.4 MBq/kg (initial burden)	single	12 - 14	Hahn et al. 1975; Mauderly et al. 1973; Henderson et al. 1978	Radiation pneumontitis; Respiratory tract tumors

Table 1.9 Studies that exposed dogs to radiation via inhalation (continued).

Radionuclide	Mode of exposure	Dose/Treatment range	Number of exposures	Age of dogs at first exposure (months)	Reference	Major results
91Y	inhalation, insoluble	0.592 - 11.47 MBq/kg (initial burden)	single	12 - 14	Summary of results available in Thompson 1989 and Gerber et al. 1996	Radiation pneumontitis; Respiratory tract tumors
144Ce	inhalation, insoluble (fused clay)	0.21 - 1200 Gy (cumulative lung dose to death)	single	12 - 14	Hahn et al. 1973; Hahn et al. 2001; Pfleger et al. 1975; Henderson et al. 1978; Hahn et al. 1999; Benjamin et al. 1975	Respiratory tract tumors
239Pu	inhalation, nitrate, low levels	0.1 - 202 kBq (initial lung deposition)	single	17 - 23	Weller et al. 1995	Lymphopenia; Bone tumors

Table 1.10 Studies that exposed dogs to radiation via inhalation (continued).

Radionuclide	Mode of exposure	Dose/Treatment range	Number of exposures	Age of dogs at first exposure (months)	Reference	Major results
238Pu	inhalation, oxide	0.74 - more than 74 kBq/kg (terminal body burden)	single	8 - 42	Summary of results available in Thompson 1989 and Gerber et al. 1996	Bone tumors; Greater deposition in the bone
238Pu	inhalation, oxide, low levels	0.13 - 210 kBq (initial lung deposition)	single	15 - 20	Park et al. 1997; Weller et al. 1995	Lymphopenia; Bone tumors; Lung tumors
239Pu	inhalation, oxide	2.22 - 11.47 kBq/kg (initial burden)	single	12 - 43	Summary of results available in Thompson 1989 and Gerber et al. 1996	Lung tumors
239Pu	inhalation, oxide, low levels	0.014 - 210 kBq (initial lung deposition)	single	14 - 25	Park et al. 2012; Fisher and Weller 2010; Weller et al. 1995	Lymphopenia; Antibody response

Table 1.11 Studies that exposed dogs to radiation via inhalation (continued).

Radionuclide	Mode of exposure	Dose/Treatment range	Number of exposures	Age of dogs at first exposure (months)	Reference	Major results
144Ce	inhalation, soluble	0.18 - 10 MBq/kg (long term retained burden)	single	12 - 14	Hahn et al. 1997; Boecker and Cuddihy 1974; Benjamin et al. 1979; Benjamin et al. 1975	Translocation of radionuclides; Lung tumors; Liver tumors; Bone tumors
90Sr	inhalation, soluble	0.067 - 4.3 kBq/kg (long term retained burden)	single	12 - 15	Gillett et al. 1987a; Gillett et al. 1987b; Benjamin et al. 1979; Benjamin et al. 1975	Bone tumors; Lung tumors
91Y	inhalation, soluble	177.6 - 7,770 kBq/kg (initial burden)	single	12 - 15	Benjamin et al. 1979	Translocation of radionuclides

Table 1.12 Studies that exposed dogs to radiation via external gamma or X-ray.

Radionuclide	Mode of exposure	Dose/Treatment range	Number of exposures	Age of dogs at first exposure (months)	Reference	Major results
60Co	external gamma ray	3 - 540 mGy/day (dose rate)	continuous	13	Carnes and Fritz 1993; Norris et al. 1968; Seed et al. 1977; Seed and Meyers 1993; Seed et al. 2002	Hematopoietic failure; Aspermy
60Co	external gamma ray	38 - 263 mGy/day (dose rate)	terminated	12 - 24.9	Carnes and Fritz 1991	Hematopoietic failure
60Co	external gamma ray	7.5 cGy/day (dose rate)	continuous	in-utero	Seed at al. 1987	Hematopoietic failure/recovery
60Co	external gamma ray (10 min exposures)	15.6 - 88.3 cGy (total dose)	multiple	in-utero - 12	Benjamin et al. 1998a; Benjamin et al. 1998b; Garner et al. 1974; Jaenke and Angleton 1990; Jaenke et al. 1977; Lao 1998; Miller and Benjamin 1985	Shortened lifespan; Neoplasia

Table 1.13 Studies that exposed dogs to radiation via external gamma or X-ray (continued).

Radionuclide	Mode of exposure	Dose/Treatment range	Number of exposures	Age of dogs at first exposure (months)	Reference	Major results
60Co	external gamma ray (limited exposure to the heart)	36 - 52 Gy (total dose)	multiple	18	McChesney et al. 1988; Gillette et al. 1985	Myocardial damage
X-Ray	external x-ray	100 - 300 R (total exposure)	multiple	8 - 15	Andersen and Rosenblatt 1969; Andersen et al. 1961	Shortened lifespan; Neoplasia
X-Ray	external x-ray	0.21 - 1.57 Gy (total dose)	single	15 - 30	Gerhartz et al. 1982; Nothdurft 1982; Nothdurft 1984	Hematopoietic cell changes

Table 1.14 Studies that exposed dogs to radiation via external gamma or X-ray (continued).

Radionuclide	Mode of exposure	Dose/Treatment range	Number of exposures	Age of dogs at first exposure (months)	Reference	Major results
X-Ray	external x-ray (unilateral vs. bilateral)	2.1 - 3.8 Gy (total dose entrance)	single	12 - 20	Baltschukat and Nothdurft 1990; Kreja et al. 1993; Calvo et al. 1994	Hematopoietic cell changes
X-Ray	external x-ray (upper and lower body - sequential)	23.4 Gy (total dose)	multiple	12 - 17	Nothdurft et al. 1989	Hematopoietic cell changes
X-Ray	external x-ray (upper vs. lower part of body)	11.7 Gy (total dose)	single	16 - 24	Nothdurft et al. 1986; Baltschukat et al. 1989; Calvo et al. 1994	Hematopoietic cell changes

Table 1.15 Studies that exposed dogs to radiation via ingestion and miscellaneous modes of exposure.

Radionuclide	Mode of exposure	Dose/Treatment range	Number of exposures	Age of dogs at first exposure (months)	Reference	Major results
90Sr	ingestion	37 - 71,800 kBq (total ingested)	multiple	in-utero	White et al. 1993; Book et al. 1982; Nilsson and Book 1987; Raabe and Parks 1993; Dungworth et al 1969; Momeni et al. 1976; Raabe et al. 1980; Momeni 1978	Bone tumors
90Sr	subcutaneous injection	5.55 - 55.5 MBq/kg (total injected)	multiple	0 - 28.8	Summary of results available in Thompson 1989	Excessive mortality; Bone tumors; Myeloid leukemia
239Pu	subcutaneous implants	1.25 - 9.46 µCi/kg (total implanted)	single	"adult"	Dagle et al. 1984	Translocation of radionuclides
90Sr	transplacental injection	0.259 - 11.1 MBq/kg (burden at birth)	single	1 to 9 days prepartum	Summary of results available in Thompson 1989	Bone tumors

Chapter 2

The Ancestry and Genetic Structure of Free-Roaming Dog Populations in Chernobyl, Ukraine[2]

Abstract

The following chapter of this thesis presents the results of a new empirical study conducted in collaboration with the National Human Genome Research Institute's Dog Genome Project that used modern SNP genotyping at 145,435 polymorphic loci to investigate the population structure and genetic diversity of the Chernobyl canines in an attempt to understand their ancestry and evolution since the time of the disaster when this population originated. Evidence suggests that the Chernobyl dog populations experienced a relatively recent population bottleneck, potentially related to their origin after the nuclear disaster. However, the Chernobyl dog population is currently more genetically varied when compared to admixed village dogs from several neighboring countries, suggesting that the current Chernobyl populations may have descended from a highly diverse starting population or may not be exclusively descendent of pets left behind by those evacuated from the Chernobyl Exclusion Zone. Alternatively, current elevated levels of genetic variation may reflect the mutagenic impacts of the radioactive landscape in which these animals reside.

[2] Spatola, G.J., Ostrander, E.A., Parker, H.G., Hogan, A.N., and Mousseau, T.A.

Introduction

The domestic dog (*Canis lupus familiaris*) has lived alongside humans for thousands of years. Although dogs were domesticated at least 15,000 years ago (Larson et al. 2012), most modern breeds were developed within the past few hundred years through intense selective breeding for desired physical and behavioral traits (von Holdt et al. 2010; Parker et al. 2017). In contrast to modern pure breeds, village dogs are populations of unowned, free-breeding domestic dogs that exist alongside human settlements throughout the world.

Most modern pure breeds have European ancestry, although several dog breeds have been identified as having more “ancient” basal positions in lineages and phylogenetic analyses (vonHoldt et al. 2010; Larsen et al. 2012; Shannon et al. 2015; Parker et al. 2017). The degree to which these breeds are supported as basal nodes on phylogenetic trees is related to the level of historic admixture within the population, with more thorough mixing corresponding to comparatively shallow basal positions (Larsen et al. 2012). Admixture is the product of interbreeding that occurs between genetically distinct populations, such as mating between dogs of different breeds.

Genetic clustering analyses reflect varying degrees of admixture within village dog populations with less foreign admixture often relating to greater proportions of regionally distinct ancestry (Boyko et al. 2009; Brown et al. 2011; Shannon et al. 2015). Regionally distinct ancestry is maintained through isolation of such populations from those that were heavily influenced by modern breed creation, particularly dogs of European descent. Village dog populations with less

modern breed admixture tend to have increased genetic diversity as a result of free-breeding and large effective population sizes. Although, some have experienced genetic bottlenecks unrelated to artificial selective pressures causing diversity within village dog populations to vary (Larsen et al. 2012; Shannon et al. 2015).

A particularly unique group of village dogs occupies the site of the historic Chernobyl nuclear disaster of 1986 and surrounding area. These free-roaming dogs are thought to be descendants of pets left behind during the evacuation of cities and towns following the nuclear accident. Because of their close proximity to the source of the explosion within the Chernobyl Nuclear Power Plant (CNPP) and subsequent nuclear fires that burned for 10 days after, the pets left behind would have certainly been exposed to very high levels of radiation. The effects of exposure to high doses of ionizing radiation are well documented (UNSCEAR 2000), and many pets left behind would have died from radiation sickness or as a result of fires that burned after the disaster. Pets that withstood these initial effects of the nuclear disaster were actively hunted by teams of men under the orders of the Ukranian Ministry of Internal Affairs, who feared that the irradiated animals would further spread nuclear contamination from their fur, and potentially rabies or other diseases, to humans they came into contact with. Although the majority of pets were likely killed, some dogs were thought to have evaded hunters by escaping beyond the Chernobyl Exclusion Zone (CEZ) where they were fed by camps of liquidators (Higginbotham 2019).

Though relatively abundant in recent years with population sizes approaching 1,000, free-breeding dogs inside the CEZ have never been studied despite their potential utility as a model for the effects of radioactive pollutants on mammals. By definition, this has immediate relevance for human populations similarly affected by the disaster in this region. As a first step towards a larger investigation of the long-lasting impacts of the Chernobyl disaster on these animals, we investigated the ancestry of dogs occupying the CEZ as it relates to their origin in this region. More specifically, we evaluated the population structure and genetic diversity of dogs from sampling locations surrounding two major population centers that were evacuated and compared them to admixed village dogs from countries near Ukraine, including Croatia, Bosnia, and Turkey.

We hypothesized that few abandoned pets in Chernobyl would have likely survived after the nuclear disaster because of exposure to high levels of radiation, fires, and subsequent hunting of the dogs, creating a bottleneck effect. A small group of founders would enhance genetic similarity among the various populations that occupy areas within the CEZ, despite free-breeding for approximately thirty generations since the nuclear disaster, likely causing them to be less diverse than the groups of admixed village dogs with no recent history of population bottlenecks. In addition, free-breeding does not necessarily mean random breeding, and genetic drift within these small populations would also contribute to lower effective population sizes and reduced heterozygosity (Kliman et al. 2008). Runs of homozygosity (ROHs) within the genome reflect past population dynamics, with longer and more frequent segments relating to a

recent contraction of effective population size (Kirin 2010). The magnitude of the ROH signal is dependent on the length and severity of the bottleneck event. In the present study, we analyzed ROHs in the genomes of Chernobyl dogs in order to test our hypothesis that the present populations in Chernobyl resulted from a bottleneck event related to the nuclear disaster. We used a Principal Component Analysis (PCA) based on a variance-standardized relationship matrix to identify variance due to population structure within the Chernobyl dogs and compared them to admixed village dogs from Bosnia, Croatia, and Turkey, allowing us to draw conclusions about the level of genetic diversity within Chernobyl populations.

If free-roaming dogs within the CEZ descended from abandoned pets that were selectively bred prior to the nuclear disaster 34 years ago, then they will likely show genetic signatures of recent admixture as a result of interbreeding since the disaster. We used the statistical software program ADMIXTURE to identify genetic admixture within the Chernobyl dogs and to further compare their individual and population genetic structures. Included in this analysis are representatives from 23 previously identified modern breed clades (Parker et al. 2017). Breed representatives are included in our analysis of genetic admixture as a means to explore potential breed ancestry of the Chernobyl dogs, as they are thought to be descended from pets. In addition, shared ancestry between Chernobyl and purebred dogs may indicate the types of dogs owned by those who were evacuated. Should admixture with breed clade representatives be

detected, this would provide future direction for more specific analyses of breed ancestry that should include more individuals from all breeds within the clade.

Methods

Sample collection, DNA extraction, and SNP genotyping

A modified version of the “Ammonium Acetate Precipitation Protocol for DNA extraction from bird blood/animal tissue” provided by Deborah Dawson from the Biomolecular Analysis Facility at Sheffield was used to extract DNA from Chernobyl dog blood samples preserved in ethanol. Chernobyl dog DNA samples were genotyped at the Ostrander laboratory at the National Institutes of Health (NIH) using Illumina CanineHD SNP arrays following standard protocols (Illumina, Inc., San Diego, California, USA). No animals were sacrificed for the purpose of this study. Samples were collected in conjunction with an ongoing spay-neuter-vaccination clinic organized by the Clean Futures Fund (CFF+). Dogs were captured by veterinarians and qualified CFF+ volunteers using humane chemical sedation and mechanical techniques, again reducing unnecessary stress to the animals when possible. Using both chemical and mechanical capture techniques reduced the effects of sampling bias by allowing us to capture more fearful individuals from a distance. Following capture and anaesthetization for surgery, blood samples were collected either via catheter or using a capillary tube to draw blood exposed from the surgery by a licensed veterinarian or veterinary technician. The 88 Chernobyl dog blood samples used in this study were collected in 2018 from areas surrounding two veterinary clinic locations set up by CFF+ inside the CEZ. The first clinic location was situated

inside the industrial areas surrounding the power plant (CNPP) and the second was approximately 22 kilometers away from the first, in an area known as Chernobyl Town (CT) (Figure 2.1). Thirty-nine dogs were captured inside the industrial areas (CNPP) and 49 dogs were captured near CT.

Village dog samples used in this study were from three countries near Ukraine, including three from Bosnia, six from Croatia, and 13 from Turkey; all of which were previously determined to be non-indigenous village dogs of predominantly European descent (Shannon et al. 2015). Village dogs from Bosnia and Croatia were sampled from various cities within the countries. Village dogs from Turkey were sampled within the cities of Istanbul and Giresun. Samples were provided from the Cornell Veterinary Biobank (Shannon et al. 2015). Genomic data from the village dogs and representatives of twenty-three previously identified breed clades used in this study were provided by Dr. Ostrander's laboratory, which heads the NHGRI's Dog Genome Project (Parker et al. 2017). The list of breeds included in this study and their corresponding clades (Parker et al. 2017) are outlined in Figure 2.2.

Data filtering and analyses

The genotypes from 88 Chernobyl dogs, 22 village dogs, and 23 pure breed representatives were filtered in PLINK 1.9 (Chang et al. 2015). Variants and samples with missing call rates exceeding 5% were discarded. One CNPP dog was excluded from the final dataset due to insufficient genotype data. Loci mapping on chromosomes X and Y were removed following genotyping and quality cleaning steps.

The Principal Component Analysis (PCA) was conducted using genotypes from 87 Chernobyl and 22 village dogs in PLINK 1.9 (Chang et al. 2015). We retained 109 samples that were successfully genotyped with a call rate equal to or exceeding 95% at 145,544 autosomal single nucleotide polymorphisms (SNPs).

Our admixture analysis dataset includes 132 samples (87 Chernobyl dogs, 22 admixed village dogs, and 23 pure breed representatives) that were successfully genotyped with a call rate equal to or exceeding 95% at 145,544 autosomal SNPs. This dataset was further filtered to leave only independent loci in approximate linkage equilibrium. Removing highly correlated loci that are likely to be in linkage disequilibrium (LD) is recommended prior to using the ADMIXTURE software, as this ancestry estimation model assumes linkage equilibrium among markers (Alexander et al. 2009; Alexander and Lange 2011). SNPs with pairwise genotypic associations of r^2 exceeding 0.25 calculated along sliding windows of 50 SNPs were discarded. A subset of 54,530 SNPs (37%) was obtained after LD pruning.

We used a cross-validation procedure built into the ADMIXTURE software to estimate the K value for which the model had the best predictive accuracy (Alexander and Lange 2011). The K values representing the most likely number of ancestral genetic lineages (populations), had the lowest standard errors of cross-validation estimates. Model evidence in this procedure is judged by the predictive accuracy of systematically masking data points, where the K value at which masked data points were most accurately predicted corresponds to the

most likely value of ancestral genetic clusters. Cross validation errors were estimated for K values of one through 27, where error values failed to further decline.

We used PLINK 1.9 (Chang et al. 2015) to calculate rates of heterozygosity and identify runs of homozygosity (ROHs) in ten randomly selected unrelated individuals from both Chernobyl subpopulations and from the population in Turkey. Because these analyses of heterozygosity and homozygosity are dependent on sample size (Pilot et al. 2015), we excluded village dogs from Croatia and Bosnia. Only three dogs were sampled from Bosnia and six were sampled from Croatia, which is not enough to produce accurate representative estimates from these populations. In addition, we only used unrelated samples for this analysis to reduce bias in homozygosity estimates. In the dataset of all Chernobyl dogs and village dogs from Turkey, we used the tag --genome to estimate genomic proportions that were identical by descent (IBD) between individuals. We removed samples with IBD proportions greater than 50%, then removed samples whose IBD proportions were outliers in the distribution of remaining samples. Ten was the largest number of samples possible to use after filtering while maintaining equal sample sizes necessary for this analysis. We identified ROHs longer than 100 kb and spanning at least 25 SNPs in individuals from each Chernobyl subpopulation and from the Turkey village dog population using the LD pruned dataset, in order to separate ROHs related to autozygosity from those related to strong LD (Pilot et al. 2015). We analyzed ROHs related to recent inbreeding rather than LD in order to target

potential effects of the bottleneck event rather than more distant population dynamics.

Fst estimates were calculated using the tag --fst specifying “within family” in PLINK 1.9 to calculate population differentiation between Chernobyl subpopulations and the Turkey village dog population. The case control tag was used to calculate differentiation between subpopulations within the CEZ.

Results

Rates of heterozygosity and population differentiation

The mean rates of heterozygosity within the CT and CNPP sampling sites were 0.372 ± 0.016 and 0.378 ± 0.012, respectively. The rates were not significantly different ($p = 0.810$; t-test). The mean rate of heterozygosity for Turkey village dogs was 0.379 ± 0.007, which was not significantly different from rates for either Chernobyl subpopulation ($p = 0.728$ and 0.896; t-tests). This suggests that all groups considered have relatively similar levels of individual genetic diversity. However, similarity in rates of heterozygosity may not be informative as heterozygosity tends to be insensitive to the effects of short-term population bottlenecks (Allendorf 1986).

The Turkey village dog population and the CNPP subpopulation were more highly differentiated based on Wright’s weighted Fst estimates compared to the Turkey village dog population and the CT subpopulation (Figure 2.10). The Chernobyl subpopulations were least differentiated, suggesting that there is considerable interbreeding between subpopulations. Alternatively, this may

suggest that the dogs currently occupying the CEZ may have descended from the same starting population.

Principal Component Analysis

As expected, principal component one (PC 1) and principal component two (PC 2) encompassed the greatest amount of variation detected across all loci (7.9% and 6.1%, respectively). The samples spread across the PC 1 axis with ranges of 0.297411 for the CNPP subpopulation, 0.338806 for the CT subpopulation, and 0.0497178 for the village dog populations collectively (Figure 2.3). The ranges of spread across the principal component two (PC 2) axis were 0.436711 for the CNPP subpopulation, 0.331858 for the CT subpopulation, and 0.0101874 for the village dog populations (Figure 2.3). The Chernobyl dogs were spread more widely across both PC axes than village dogs from Turkey, Bosnia, and Croatia, indicating that genetic diversity was greater within the Chernobyl populations. In addition, the Chernobyl dogs did not appear to cluster based on geographic location. Despite being from three different countries, the village dogs all clustered together. Although, one village dog from Turkey and another from Croatia separated from the rest of the village dog cluster, reflecting some degree of genetic variation within this group. This variation is not surprising, as they were previously found to be of admixed European ancestry and were sampled from different countries (Shannon et al. 2015). Overall, it is clear that genetic variation within the Chernobyl population is greater than variation within the group of village dogs from countries near Ukraine, suggesting that the Chernobyl dogs come from diverse ancestral backgrounds.

Dogs from Chernobyl Town appeared more diverse across PC 1 compared to those from the industrial areas (CNPP), and this principal component encompassed a greater amount of variation detected across all loci (7.9%) (Figure 2.3). Dogs captured within the industrial areas (CNPP) spanned a greater distance across PC 2, which took up a larger range compared to points across the PC 1 axis. Therefore, the amount of genetic variation within each CEZ subpopulation did not appear significantly different. There was some separation of the Chernobyl subpopulations across PC 1, although many samples were intermixed between the two larger clusters. This indicates that there is shared ancestry among dogs from either capture location, which is likely a result of gene flow between subpopulations. On the PC 2 axis, there are smaller clusters of nine and seven CNPP dogs that fall between the ranges of approximately 0.19 to 0.25 and -0.19 to -0.25, respectively. These groupings may be indicative of familial clusters, potentially related to popular sires.

Cross-validation procedure

K = 5 and K = 7 had cross-validation standard errors of 0.52483 and 0.52491 respectively, which were the lowest cross-validation standard errors for the number of potential ancestral populations of one through 27 that were tested (Figure 2.4). Figure 2.5 and Figure 2.6 show results from the ADMIXTURE analysis with the plots representing K = 5 and K = 7, respectively. Trends in genetic diversity and structure that are discussed in the ADMIXTURE results were consistent for K estimates four through nine (Figure 2.7).

ADMIXTURE results

The Chernobyl dog populations are clearly separated from most of the purebred and village dogs, reflecting differences in ancestry and levels of admixture (Figures 2.5, 2.6, and 2.7). The red ancestral fractions represent the single genetic cluster that was largely shared by pure breeds and village dogs from Croatia, Bosnia, and Turkey. Genetic differences that cause diversity between pure breeds were not detected. In addition, variation in levels of admixture between most village and pure-bred dogs appeared to be similar, which indicates that the analysis was insufficient to compare admixture levels related to more distant introgression. Genetic variation that was detected by this analysis explained diversity among dogs from the Chernobyl populations and is largely represented by blue ancestral fractions. Different shaded blue ancestral fractions represent different genetic clusters that account for variation among dogs from the Chernobyl groups, potentially showing different ancestral lineages stemming from distinct populations that were not included in this analysis.

Three individual samples from the village dog group and one purebred sample shared larger portions of genetic ancestry with the Chernobyl groups than the rest of the dogs from either the village or purebred groups (Figures 2.5, 2.6, and 2.7). The purebred dog that had a large portion of shared ancestry (>90% at both optimal K values) with the Chernobyl dogs was the German shepherd, which represented the New World clade of modern breed dogs in this study (Figures 2.5 and 2.6). The separation of this dog from the rest of the pure-bred dogs indicates that the Chernobyl dogs have recent ancestry stemming

from this breed or modern breed clade. The three village dogs that had larger proportions of shared genetic ancestry with the Chernobyl dogs were from each of the three nearby countries that these dogs were sampled from (Croatia, Bosnia and Turkey). Their total proportions of shared ancestry with Chernobyl dogs were between 24% and 76% for K = 5 (Figure 2.5) and between 29% and 79% for K = 7 (Figure 2.6). For both K estimates of five and seven, a single village dog from Turkey appeared to derive more than 74% of its ancestry from genetic clusters associated with the population structure of the Chernobyl dogs, suggesting that this dog shares recent genetic ancestry with dogs occupying the CEZ (Figures 2.5 and 2.6).

The genetic structures of individual Chernobyl dogs were varied, suggesting that admixture levels vary between individuals within the CEZ populations (Figures 2.5 and 2.6). The Chernobyl dogs had inconsistent proportions of ancestral fractions, and the number of ancestral clusters represented in individual dogs was also inconsistent. Some individual samples from the Chernobyl population had proportions from a single ancestral cluster span the entire length of their genetic ancestry, reflecting a lack of admixture, while others had different proportions of ancestry from all of the ancestral clusters that were identified in the analysis, showing high levels of admixture (Figures 2.5, 2.6, and 2.7). This inconsistency reflects increased genetic diversity within the Chernobyl metapopulation.

All of the clusters that explained genetic diversity within the Chernobyl metapopulation (blue shaded ancestral fractions) were represented to some

degree in both subpopulations (Figures 2.5, 2.6, and 2.7). This suggests that the dogs from both Chernobyl subpopulation have shared ancestry. However, the proportions of ancestry from some genetic clusters were greater and more frequent in one subpopulation over another, showing genetic differentiation of the CEZ subpopulations. For example, in the analysis where K was set to five, the ancestral cluster represented by the lightest blue colored fractions was found in 22 CT dogs at a proportion of over 50% (Figure 2.5). In the CNPP subpopulation, only two dogs had ancestry proportions over 50% from this genetic cluster (Figure 2.5).

Dogs from the CNPP location had average ancestral proportions of 5% and 3% from the genetic cluster that was largely shared by village and purebred dogs (red ancestral fractions) for K = 5 and K = 7, respectively (Figures 2.5 and 2.6). Dogs from the CT location had average ancestral proportions of 16% and 13% from this genetic cluster for K = 5 and K = 7, respectively (Figures 2.5 and 2.6). Some Chernobyl dogs had long runs of red ancestral fractions, more similar to the genomic structures of the modern pure breeds and village dogs. In the analysis where the K estimate was set to five, 18 dogs from the CT subpopulation had red ancestral proportions greater than 20%, while only five dogs from the CNPP had equivalent proportions of ancestry from this cluster (Figure 2.5). In addition, four dogs from the CT subpopulation had red ancestral proportions greater than 50% for this analysis (Figure 2.5). This indicates that their genetic ancestry is different from the majority of dogs within the Chernobyl populations, and may show a stronger influence of recent migrants. Greater

proportions of unresolved ancestry in the CT dogs suggest that there is greater non-local genetic flow into this subpopulation.

Runs of homozygosity (ROHs)

A greater number of ROH segments were identified in dogs from the CNPP sampling site compared to dogs from the CT sampling site, although both Chernobyl subpopulations showed an abundance of ROH segments compared to the village dogs from Turkey (Figures 2.8 and 2.9). This suggests that the Chernobyl populations have smaller effective population sizes (Ceballos et al. 2018), with the CT subpopulation being intermediate in size to the CNPP subpopulation (smallest) and Turkey village dog population (largest). In addition, the Chernobyl dog populations had higher frequencies of ROH segments greater than 2 Mb when compared to the Turkey population (Figure 2.8). This is consistent with our expectations and reflects a recent bottleneck event within the CEZ populations, potentially related to events following the nuclear disaster.

Discussion

If the Chernobyl dogs were descendants of a small group of pets left behind after the nuclear disaster, the bottleneck that occurred around the time of the disaster would have influenced the level of genetic similarity between members of the current metapopulation, approximately thirty generations later. Rates of heterozygosity within the Chernobyl subpopulations were not significantly different from that of the Turkey village dog population. In addition, the Chernobyl subpopulations did not appear to be highly differentiated based on Fst estimates and did not cluster in the PCA based on geographic location,

suggesting that there is interbreeding between the subpopulations. Because there does not appear to be an excess or lack of heterozygosity among either of the Chernobyl subpopulations and the subpopulations do appear to be highly differentiated, dogs from either capture location may be considered as members of a larger CEZ metapopulation.

Elevated levels of genetic variation were found within Chernobyl dog populations when compared to other groups of village dogs. Despite having compared the Chernobyl dogs to village dogs from three different countries, the greatest amount of variation detected in both the PCA and ADMIXTURE analyses was explained by differences between dogs from the Chernobyl populations (Figures 2.3, 2.5, and 2.6). This is especially unexpected given that the Chernobyl dogs were captured from locations no more than 22 kilometers apart. This is a sampling distance significantly shorter than that which spans Turkey, Croatia, and Bosnia. Nevertheless, dogs from Chernobyl showed more significant within population variation, demonstrating elevated genetic diversity within the current CEZ population. This suggests that the current CEZ populations may have descended from more highly diverse starting populations, causing them to appear more diverse than the village dog populations we compared them to.

Previous studies showed that the village dogs from Croatia, Bosnia, and Turkey were admixed compared to indigenous populations of village dogs (Shannon et al. 2015). The dogs from Chernobyl had widely varied ancestral proportions compared to most of the other village dogs included in this study,

which had largely consistent proportions of ancestry (Figures 2.5, 2.6 and 2.7) showing more recent and extensive admixture within the Chernobyl populations. Because the Chernobyl populations were expected to have originated after the nuclear disaster only 34 years ago, the admixture detected likely reflects their recent origin from a diverse group of founders. In addition, the ROH analysis revealed genetic signatures of a relatively recent population bottleneck, further supporting the inception of dog populations in the CEZ following the nuclear disaster. Alternatively, elevated ROH statistics could be indicative of recent inbreeding, however rates of heterozygosity within the subpopulations appeared to be comparable to that of the Turkey village dog population, suggesting that inbreeding is unlikely to be the cause of increased ROH within the Chernobyl metapopulation.

Some Chernobyl dogs had large proportions of ancestry from the genetic cluster that was shared by most pure-bred dogs and village dogs from Croatia, Bosnia, and Turkey (Figures 2.5, 2.6, and 2.7), potentially showing the influence of migration into the CEZ, as these dogs had less shared ancestry with the majority of Chernobyl dogs. Humans often transport dogs of different ancestry to areas already colonized by local populations of free-roaming dogs (Boyko et al. 2009) and admixture with the foreign dogs increases overall variation within the village dog populations (Larsen et al. 2012, Shannon et al. 2015). It is possible that recent migrants contributed new genetic material to the existing gene pool since the disaster, further increasing variation within the populations that exist in the CEZ today.

Recently people have moved back to less contaminated areas that were evacuated after the nuclear disaster, such as Chernobyl Town. Pet dogs brought in by new inhabitants may have contributed genetic variation to the existing population of free ranging dogs, causing the effective population size of the CT subpopulation to appear larger than that of the CNPP subpopulation. More dogs from the CT subpopulation had large proportions of ancestry that were different from the majority of Chernobyl dogs and more similar to the genetic structures of pure-bred dogs and admixed village dogs from Bosnia, Croatia, and Turkey. It is possible that these individuals are more closely related to pet dogs brought into the area by current residents.

Including village dogs from three different neighboring countries provides a weak comparison of population structure between admixed village dog populations because they are not from the same local genetic pool. Including mixed breed dogs from local shelters may provide a more informative comparison. Although, the geographically separated village dogs we used for comparison to the Chernobyl population were genetically similar to one another relative to the diverse dogs occupying the CEZ, so this does not affect our conclusions regarding genetic diversity within the Chernobyl dog populations. In addition, this study only included 23 of over 350 modern pure breeds that exist, which is informative but far from an exhaustive analysis. The inclusion of more modern breeds in future analyses, particularly from the New World clade of domestic breeds, will be useful in further investigating the ancestry of the Chernobyl population. Additionally, more studies are required to determine the

exact timing and cause of the apparent bottleneck(s), as this could be related to a number of events. For example, it is unclear whether the bottleneck occurred as a result of the nuclear contamination, fires, hunting of the dogs, or events predating the nuclear disaster.

Future studies should seek to better understand the source of the extensive genetic variation found within the Chernobyl dog populations as it may pertain to a variety of evolutionary mechanisms influencing them. Differentiating between mechanisms that leave similar genetic signatures is complex. In addition to admixture, genetic mutation has the potential to increase diversity within a population. The Chernobyl dogs reside in an area contaminated by ionizing radiation, which is a well-known mutagen (Muller 1950), providing future direction for understanding the source of diversity found within this unusual village dog population, particularly as it may pertain to regions of the genome undergoing selection associated with pressures from exposure to radiation.

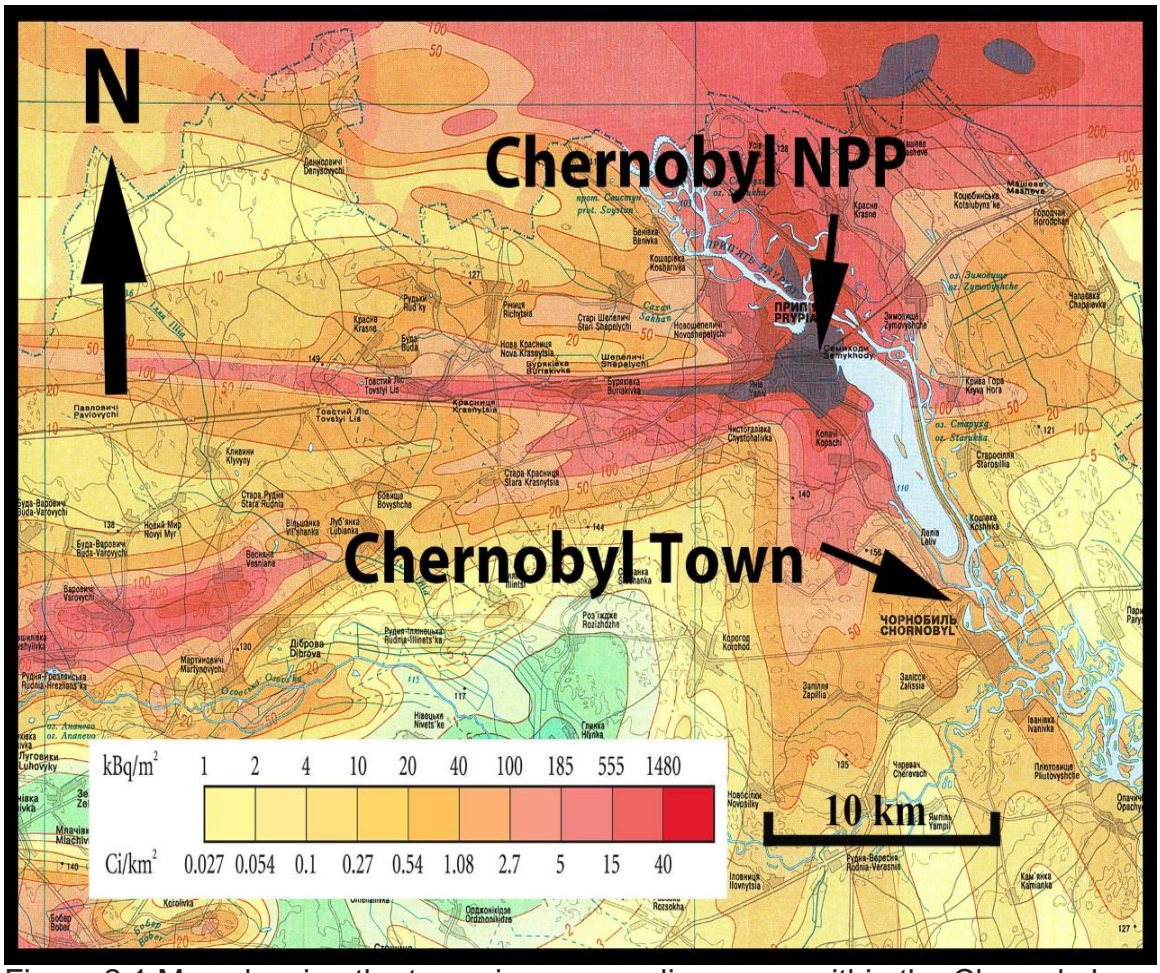

Figure 2.1 Map showing the two primary sampling areas within the Chernobyl Exclusion Zone. Adapted from Shestopalov 1986.

Breed	Abbreviation	Clade
American Cocker Spaniel	ACKR	Spaniel
Akita	AKIT	Asian Spitz
Basset Hound	BASS	Scent Hound
Briard	BRIA	Continental Herder
Brussels Griffon	BRUS	Toy Spitz
Chihuahua	CHIH	American Toy
Golden Retriever	GOLD	Retriever
German Shepherd Dog	GSD	New World
German Shorthaired Pointer	GSHP	Pointer Setter
Icelandic Sheepdog	ICES	Nordic Spitz
Irish Terrier	IRIT	Terrier
English Mastiff	MAST	European Mastiff
Miniature Pinscher	MPIN	Pinscher
Miniature Schnauzer	MSNZ	Schnauzer
Pomeranian	POM	Small Spitz
Puli	PULI	Hungarian
Rat Terrier	RATT	American Terrier
Rottweiler	ROTT	Drover
Saluki	SALU	Mediterranean
Shih Tzu	SHIH	Asian Toy
Standard Poodle	SPOO	Poodle
Shetland Sheepdog	SSHP	UK Rural
Saint Bernard	STBD	Alpine

Figure 2.2 Table of modern breed representatives from twenty-three established clades.

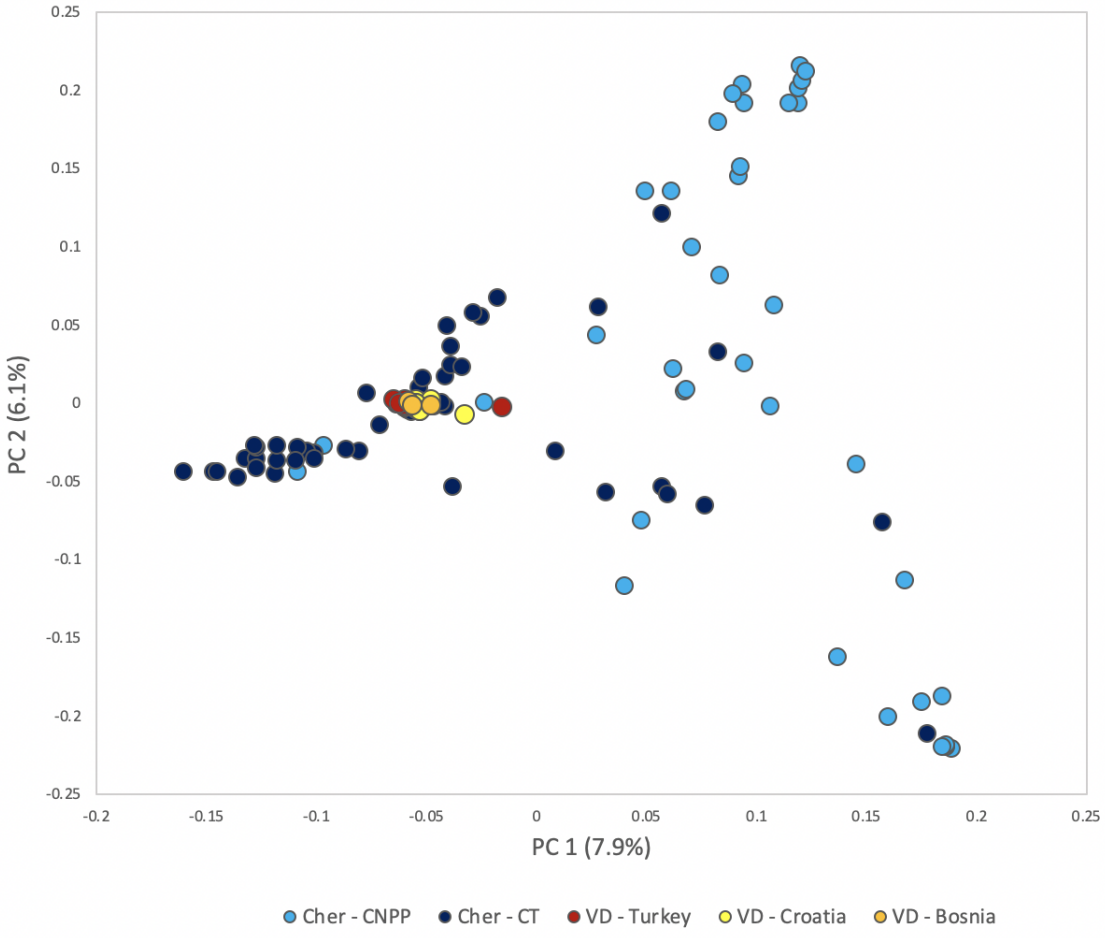

Figure 2.3 Results from exploratory principal component analysis (PCA) computed in PLINK 1.9. PCA includes the CEZ population (CNPP; light blue circles, CT; dark blue circles) and village dogs from countries near Ukraine (Turkey; red circles, Croatia; yellow circles, Bosnia; orange circles).

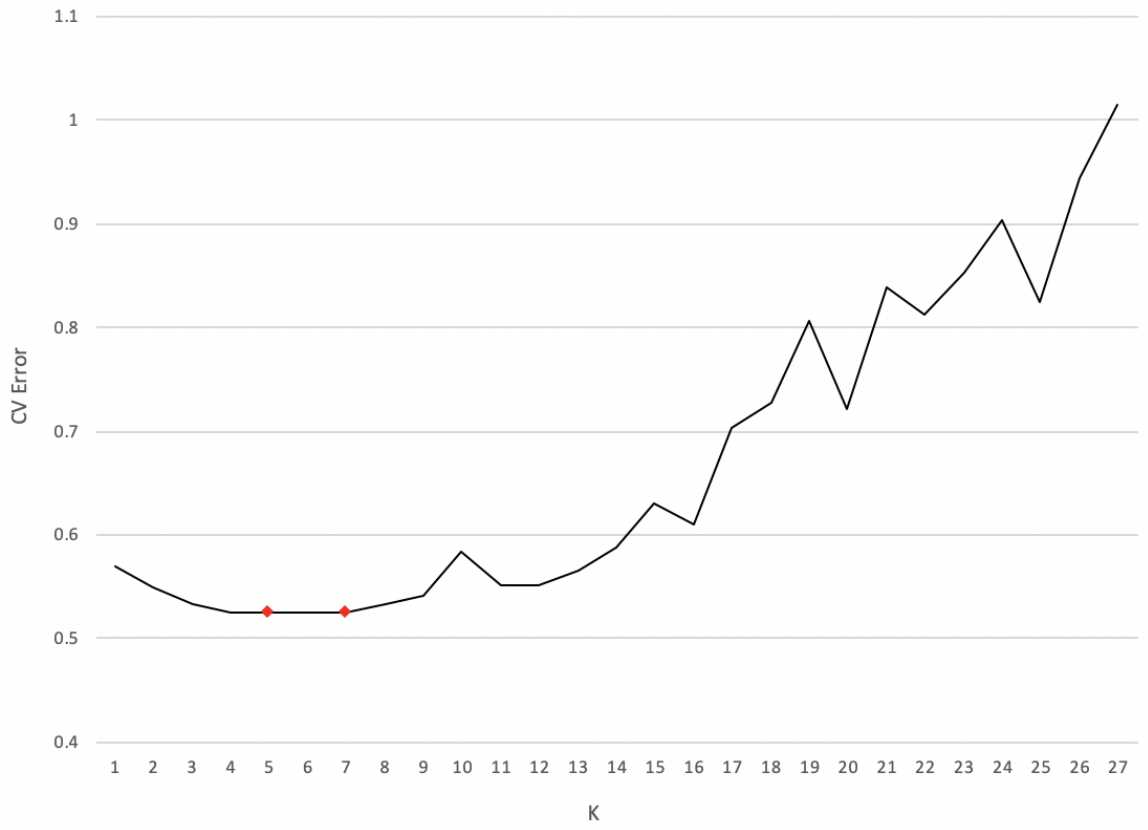

Figure 2.4 Cross validation plot showing the standard errors of cross validation estimates that represent the most likely numbers of ancestral genetic clusters.

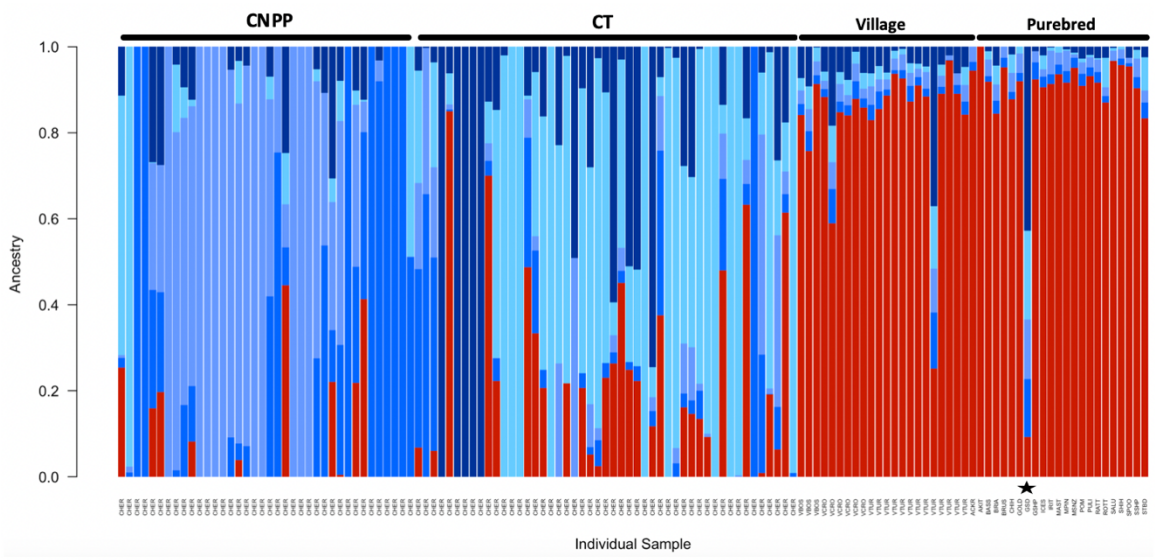

Figure 2.5 ADMIXTURE analysis results for K = 5. Black bars indicate the individual samples belonging to each current population and populations are labeled above. Individual samples are labeled on the x-axis beneath the plot. The black star on the horizontal axis of individual labels identifies the German shepherd breed sample representing the New World clade. The y-axis indicates the length of ancestral proportions.

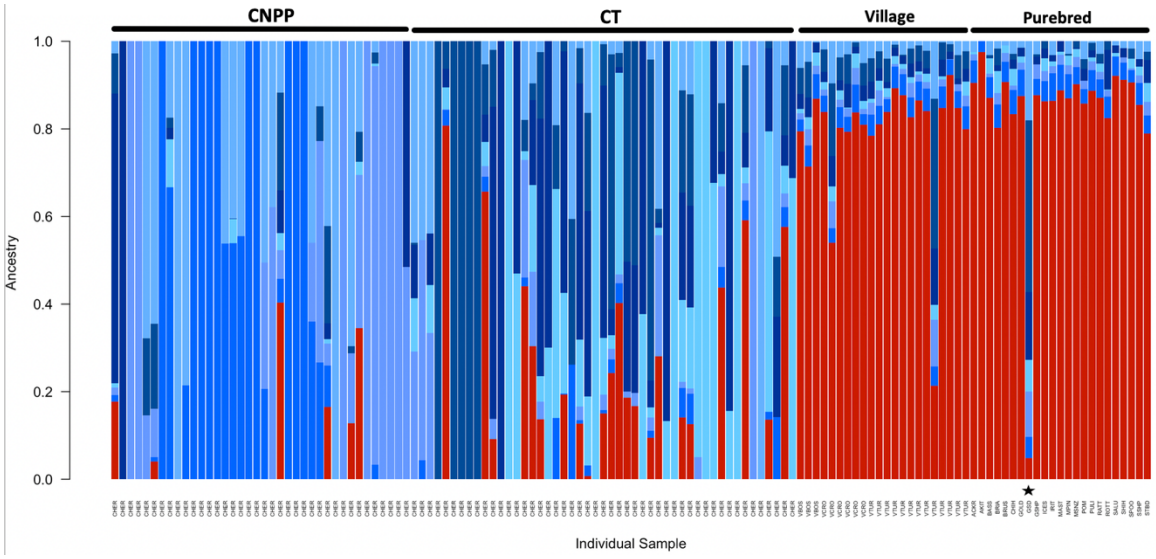

Figure 2.6 ADMIXTURE analysis results for K = 7. Black bars indicate the individual samples belonging to each current population and populations are labeled above. Individual samples are labeled on the x-axis beneath the plot. The black star on the horizontal axis of individual labels identifies the German shepherd breed sample representing the New World clade. The y-axis indicates the length of ancestral proportions.

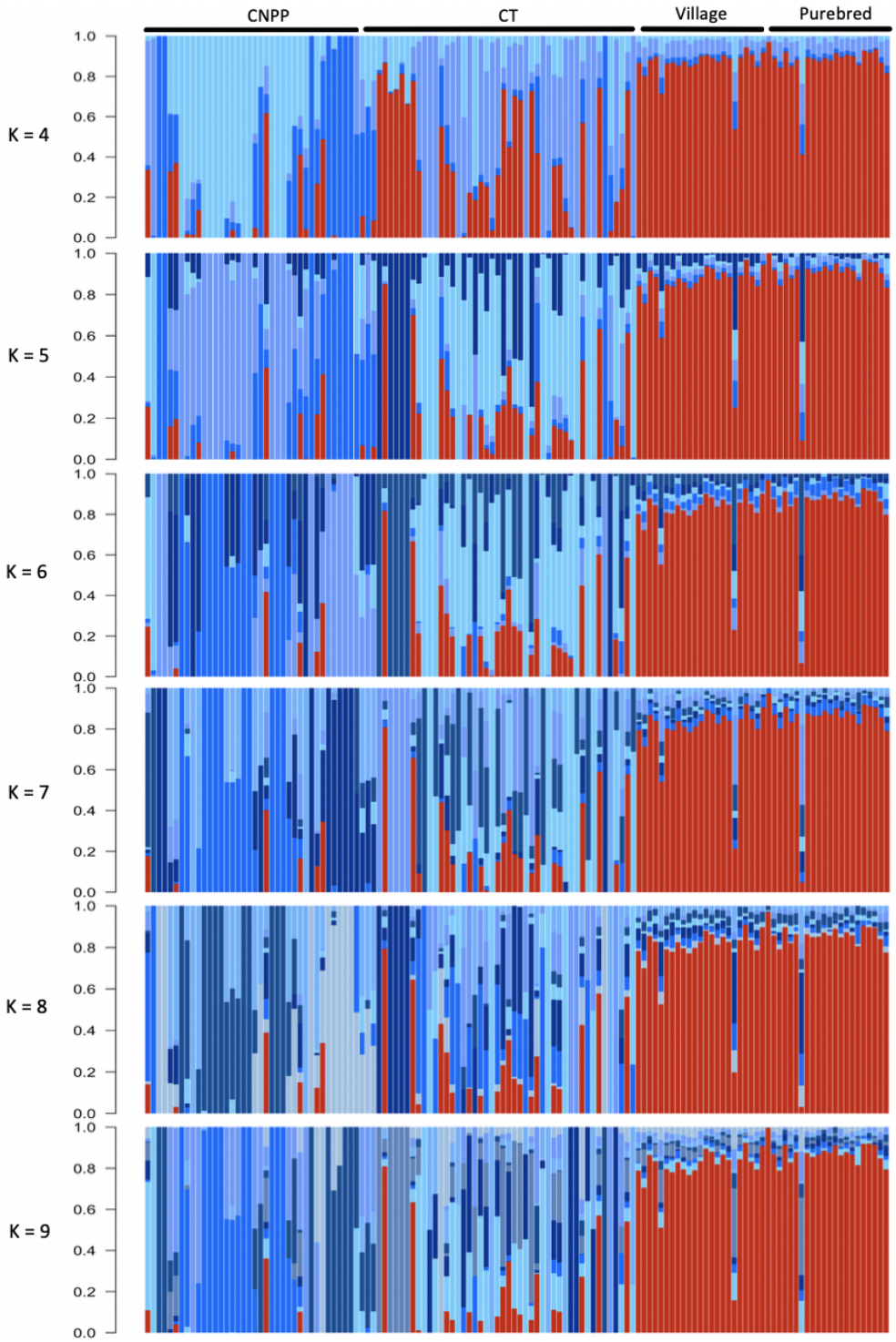

Figure 2.7 ADMIXTURE analysis results for K estimates four through nine. Black bars indicate the individual samples belonging to each current population and populations are labeled above. The y-axis of each plot indicates the length of ancestral proportions.

Location	Total number of ROHs	Total length of ROHs (Mb)	Number of ROHs <1Mb	Freq. of ROHs <1 Mb	Number of ROHs 1-2 Mb	Freq. of ROHs 1-2 Mb	Number of ROHs >2 Mb	Freq. of ROHs >2 Mb	Largest ROH length (Mb)
TURK	63	456	1	0.016	18	0.286	44	0.698	47
CT	123	1363	1	0.008	15	0.122	107	0.870	57
CNPP	208	1910	1	0.005	30	0.144	177	0.851	54

Figure 2.8 Frequencies and lengths of runs of homozygosity (ROHs).

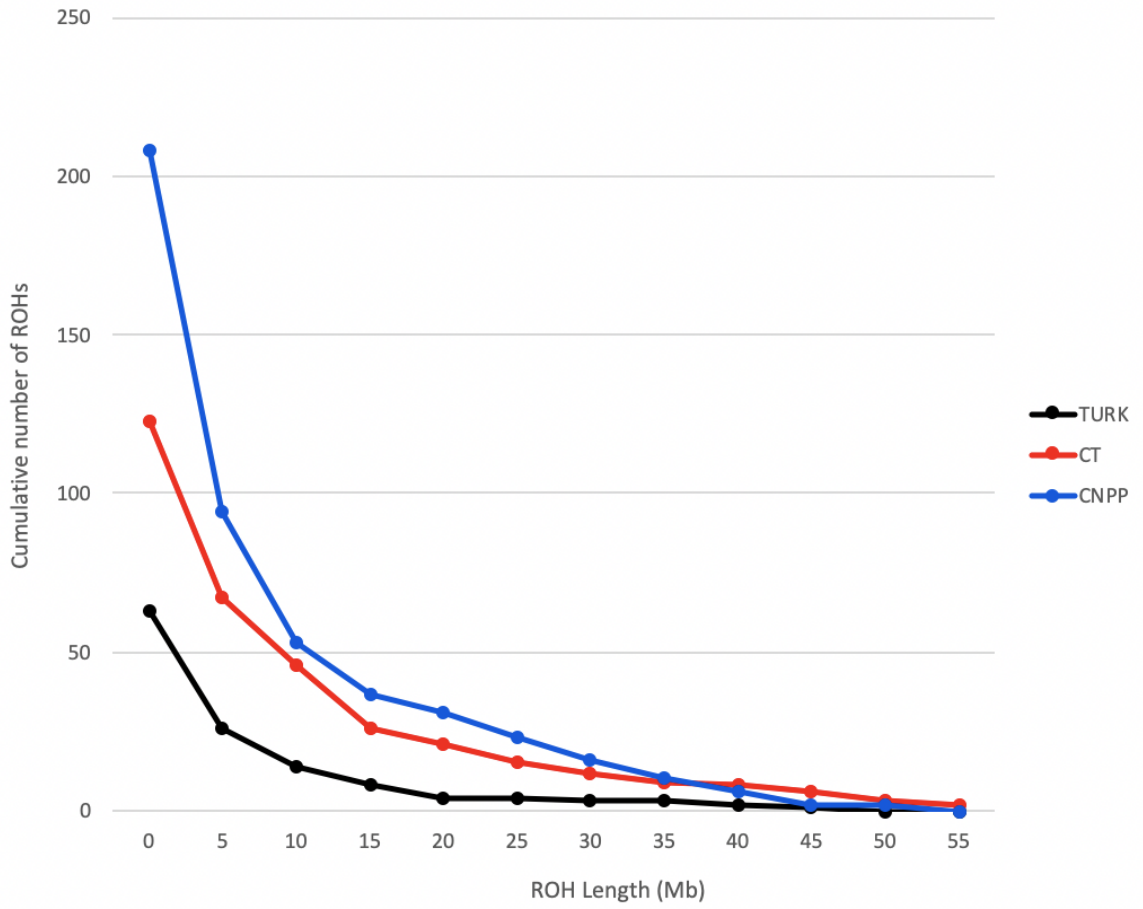

Figure 2.9 Frequency distribution of runs of homozygosity (ROHs). The y-axis represents the cumulative number of ROHs of length equal to or greater than the length indicated on the x-axis.

	CT
CNPP	0.018

	TURK
CNPP	0.059
CT	0.025

Figure 2.10 Wright's weighted Fst estimates computed in PLINK 1.9.

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