UNDERSTANDING WOLF HOWLS AND THEIR APPLICATION IN INDIVIDUAL IDENTIFICATION AND POPULATION ESTIMATION

THESIS

SUBMITTED TO THE

FOREST RESEARCH INSTITUTE DEEMED to be UNIVERSITY

DEHRA DUN, UTTARAKHAND

For

THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY IN FORESTRY

(Wildlife Science)

By

Sougata Sadhukhan

Research Center

भारतीय वन्यजीव संस्थान
Wildlife Institute of India

Year

2022

DECLARATION

I hereby declare that the thesis entitled "***Understanding wolf howls and their application in individual identification and population estimation***" submitted by myself, **Mr. Sougata Sadhukhan (Enrolment No. 16PHD411)**, to Forest Research Institute Deemed to be University, Dehradun for the award of the degree of **Doctor of Philosophy in Forestry (Wildlife Science)** is a record of original research work carried out by me under the supervision of **Dr. Bilal Habib**, Wildlife Institute of India, Dehradun and has not formed the basis for an award of any other degree or diploma. I also declare that the thesis embodies my own work, observations, and analysis, and in that respect, the investigation appears to advance knowledge in the subject.

Dehradun, the February 15, 2022

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DECLARATION

This is to certify that the thesis entitled ***"Understanding wolf howls and their application in individual identification and population estimation"*** submitted by **Mr. Sougata Sadhukhan** (**Enrolment No. 16PHD411**) to Forest Research Institute Deemed to be University, Dehradun (FRI), For the award of the degree of **Doctor of Philosophy in Forestry (Wildlife Science)** is a record of bonafide research work carried out by him, under my supervision. The thesis has been duly checked through 'URKUND', a plagiarism detection tool approved by FRI. Deemed to be University and the thesis has plagiarism to acceptable limits. No part of this thesis has been submitted for any other degree/diploma of the same institution where the work is carried out or to any other institution. It fulfills all the requirements of the ordinance governing the award of a Ph.D. Degree of FRI, Deemed to be University, Dehradun. Mr. Sougata Sadhukhan has adequate attendance during his thesis work, and he was not engaged in any paid assignment.

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Sub: - Registration for Doctor of Philosophy Degree in Forestry.

Dear Sir/Madam,

I would like to inform you that the following decisions have been taken for your enrolment as Research Scholar for the Degree of Doctor of Philosophy in Forestry in this Institute:-

1. You have been registered for Doctor of Philosophy i.e **01.03.2017 to 31.08.2022** as PhD Research Scholar.
2. Your Enrolment number is: - **16PHD411**
   (For all further correspondence please quote your enrolment number.)
3. Name of Research Centre: - **Wildlife Institute of India, Dehradun**
4. The Topic of research approved by the FRI University: **"Understanding Wolf Howls And Their Application In Individual Identification And Population Estimation."**
5. Name of Discipline: - **Wildlife Science**
   (As per clause 3.3 of the Ph.D. Ordinance)
6. (i) Name of Supervisor : - **Dr. Bilil Habib**
   (ii) Name of Co-Supervisor: - **Nil**
7. **You are advised to deposit:-**
   (a) The next installment of Laboratory fee payable at FRIDU/Research Centre concerned through bank draft in the month of March, 2018
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(A.K. Tripathi)
Registrar
FRI Deemed University

Encl: 1. Fee receipt No. 2265 dated 01.09.2017 for Rs. 26,500/-
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Copy to the following for information and necessary action:-

1. Dr. Bilal Habib, (Supervisor of the Scholar), Scientist-E, Dept. of Animal Ecology & Conservation Biology, Wildlife Institute of India, P.O. Box No. 18, Chandrabani, Dehradun-248001.
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(A.K. Tripathi)
Registrar
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Reminiscing the journey so far...

The moment has finally arrived. Hours after days after months after years, here I am, finally, writing the last few words of my doctoral thesis. As I sit down to pen my acknowledgments, I traverse into reflections on my Ph.D. journey.

In the pursuit of my Ph.D., I have met some beautiful people whose company has never made me feel alone- neither during fieldwork nor during running some tedious code in R software. Ph.D. is a remarkable journey for every scholar, and I am no exception. Like others, mine, too, was full of ups and downs. But the presence of those special people has always kept me strong on this journey.

March 1, 2017, was when I finally registered for the Doctor of Philosophy. But this philosophical journey started long before. As a child, I saw my mother talking to our pet dogs and cats. I was intrigued by their ability to understand our language, the standard commands at least. I was surprised that despite being the most intelligent creature on earth, we could not decode their vocals. With an interest in technology, I have always dreamt of making a device to decode animal language. But during school days, it did not go beyond customising a microphone in a long wire to record the calls of dogs and cats at home and understand nothing from the recording. The project became silent as my focus shifted toward the school syllabus to qualify for examinations. After finishing school, my strong interest in animals led me to take Zoology during my under-graduation. My under-grad journey gave me the first opportunity to step into a national park. Visiting the historical Jim Corbett National Park motivated me to pursue a wildlife career.

However, the challenges before me were manifold. I became the first college graduate in the family but was unsure about higher education as our family was struggling with finances. One day, when I was sitting on the edge of my chair, checking my final undergrad marks and pondering what to do next. My mother came to me and asked me whether I wanted to pursue a Master's degree. I told her about the financial requirements even in the govt aided colleges. She told me to start my applications and assured me that she would arrange the finances. My eyes sparkled with hope. Finally, I enrolled in a Master's programme in Zoology at Serampore College. In college, I met Dr. Subhadeep Sarkar, my first academic mentor, who helped shape my thoughts and encouraged me to pursue my dream. He understood my interest in wildlife and supported me. He introduced me to Simultala Conservationists, an organisation that works for rural wildlife conservation. I met Vishal Da (Vishal Santra), the organisation's founder, and later I became part of this organisation. I am grateful to the organisation and the passionate people I met there. Dr. Sarkar encouraged me to present my work at different conferences to get exposure. When I was presenting a poster at one of the state-level conferences, an elderly person came to me and asked me to contact him after the conference. I asked him his designation; he humbly replied, "I am Dr. A. K. Ghosh, Ex-director of ZSI". And that is how I met my second academic mentor. He was one of the humble people who helped me shape for research after my M.Sc. Fearing rejection, I was nervous about appearing for an interview at my dream institution, the Wildlife Institute of India. He not only encouraged me but made efforts to groom me for the interview. Unfortunately, he is no more with us, and I am sure he would have been happy to see my thesis. I cannot thank enough Dr. Y. V. Jhala and Mr. Qamar Qureshi for trusting me and allowing me to begin the journey with WII. Entering WII for the first time as a researcher was a dream come true. That opportunity exposed me to learning about different wildlife techniques and tools. With the support of both my guides, I explored many wildlife models and analyses, which helped me grow in the process and think beyond.

During my research tenure at WII, I met Dr. Bilal Habib. As a young scientist, he had very innovative ideas for wildlife research. One Fulbright fellow, Ms. Lauren Hennelly, joined him to research wolf howls. The research was the first of its kind in WII. It rekindled my childhood curiosity to work on animal communication. When I met Dr. Habib, he informed me that a project on wolf howl was in the pipeline. I was excited and appeared for the interview. Although my interview for the esteemed project went well, the interview round was supposed to get canceled due to some administrative concerns. My dream was about to take a halt. One day, Dr. Habib called to give me some data. He gave me 15 days to learn bioacoustics and submit a report. The condition was simple yet like a gigantic mountain to climb- if I could analyse those data within the time, I would be in for the project with all the support. No one around me had recommended this arduous challenge. But I was adamant because it was the glimmer of hope on the way to my dream research. During the same time, I was also involved as an Organiser at Herp School, and many of the talks were on bioacoustics. That helped me a lot to understand the basics. I should mention the generosity of Dr. Ulmer Grafe (Universiti Brunei Darussalam), who sat with me to teach and explain the acoustics software for one and a half hour just before catching his flight. Many things, like the statistical analysis, were yet to be figured out. I came across some papers by Dr. Holly Root-Gutteridge and decided to email her seeking some guidance. She responded within a day with all the codes and explanations for my curiosity. There was still a long way to go, as all the codes were in MATLAB, a software I had never used before. However, with the help of the internet and other reading material, I was able to run the codes. But the work was not finished yet, and I had reached the 14th day. By the next morning, I had to meet two deadlines - finish writing the report and show the documentary about Herp school. I was apprehensive, but a friend came up and volunteered to accompany me that night. He was Anukul (Dr Anukul Nath). He stayed awake with me for the whole night, sharing my workload. And after several file crashes, the Herp school documentary and the bioacoustics analysis report were finished by 7:30 am on the 15th day. I submitted both of them on time. I saw a captivated smile on Bilal Sir's face when he said to me, 'welcome to the lab'. I learned a life lesson that day that nothing is impossible if there is a will, and sometimes the circumstances also help you if you want something madly. Those 15 days were the most incredible learning chapters of my life.

I started the journey of wolf research first from the captive wolves of Jaipur Zoo. The zoo authorities were accommodating and welcomed my research. Spending nights at the zoo was a lifetime experience. After recording howls from Jaipur Zoo, I reached Maharashtra. Finding wolves in the grassland was like finding a needle. Moreover, without field support and not knowing Marathi added to the challenge. Just after arriving in Maharashtra, I met Mihir Godbole from wolf gang, Pune (Presently known as Grassland Trust). He helped me figure out logistics, contacts, and, most importantly, finding wolf habitats. Besides him, I enjoyed fieldwork with Vishwatej, Ashwin, Milind, and Siddhesh. It took a lot of patience to see a wolf in the fragmented grassland of Maharashtra and endurance to record them finally in the wild. From December 2015 to July 2016, I roamed around eight different districts of Maharashtra using state buses, bikes, and trains. I found many wolf habitats around the landscape with the help of the local forest department and wildlife enthusiasts; Vinit Arora, Rajesh Pardeshi, Sawan Behkar, Saurav Sukhdev, and R.V. Kasar went out of their way to make my fieldwork successful. I am highly grateful to Shree Sunil Limaye (CCF) and Dr. Vinay Sinha (APCCF) from Maharashtra Forest Department for giving permission and for their intellectual input. During this time, I conducted nearly 200 howling survey sessions with 30 successful howling responses, including filming some rare wolf footage of preying sheep. Now, it was time to sit with the data, analyse them and present it in front of a large audience at the Internal Research Seminar at WII. During the seminar, the whole lab united and effortlessly made all the presentations successful. I thank Shivam Bhaiyan (Dr. Shivam Shotriya), without whom I would not have been able to finish the analysis on time. Our supervisor is a perfectionist, so he does not let you go until you are well enough. With all their effort, my talk went well, and I got appreciation from everyone, including our Director, Dr. V. B. Mathur. But the appreciation from perfectionists like our supervisor is always special and gives you more enthusiasm for doing something better.

On March 1, 2017, I registered for Ph.D. with the key aim of standardising the howling survey for the population estimation of wolves. We got funding from the Department of Science and Technology (DST), Govt of India, to study the movement pattern of wolves alongside the population study. Shaheer (Mr. Shaheer Khan) joined us to explore the movement pattern of wolves and later enrolled for Ph.D. in wolf ecology. His effort and support became essential for the rest of the Ph.D. timeline. Capturing a wolf in the wild is a task of endurance and perseverance. Three people (the three musketeers) joined us as field assistants in the arduous task- Daut (Daut Shaikh), Bapu (Shivkumar More), and Sarang (Sarang Mhamane). We failed to detect wolves in the first five days, and our foothold traps resulted in capturing of dogs. Resetting traps require 45mins to 1 hour. Mihir and I started visiting other places, searching for wolves, and finally detected one near Gangewadi. On December 25, we decided to shift our traps to Gangewadi. Just after setting the traps, we saw a wolf around it. But suddenly, it changed her path. Hours passed after that, and the sun was just about to set, yet success was a long way away from us. During dusk, Bilal sir saw a movement around the trap. We ran and found a wolf. We were all super excited. Now it was time to cool down and handle the animal with full responsibility. On Christmas eve of 2017, we had our first collared wolf, Merry. It took another week to collar a second wolf from the same area. Within the next three years, we collared another nine wolves around different parts of Maharashtra. All the collaring events took a good amount of examination with our patience and tenacity. I appreciate the hard work of the three musketeers (Daut, Sarang, and Bapu). Besides, we got great voluntary support from Wolf gang Pune, Rajesh Pardeshi, Sagar Kalker, Prasad, and his family. I tracked wolves in the field and started recording their howls to understand their behaviour.

I was just about to begin my howling survey on collared wolves with total capacity; Bilal sir, asked me not to go to the field unless I published the first paper. I lacked the skills and orientation to write papers. After struggling for three months, I made the first draft ready, but it was still a long way from getting published. However, it took me 8-9 months to complete revisions with help from Lauren (Dr. Lauren Hennelly) and unknown reviewers from the journal. Finally, it got published on October 31 31, 2019 – "Characterising the harmonic vocal repertoire of the Indian wolf (Canis lupus pallipes)". Although I was not happy with the decision to stop me from going to the field. Today, I understand the importance of it to overcome my fear of writing and how the first paper helped me orient to output-based research work. In 2019, I also got a chance to present at the International Bioacoustics Congress, Brighton, UK, where I met Holly and Arik (Dr. Arik Kershenbaum, University of Cambridge), with whom I connected in 2015. This interaction was essential to take my study one step forward. Throughout my Ph.D. journey, Holly played a significant role in mentoring me.

I am grateful to Dr. Holger Klink (Director, K. Lisa Yang Center for Conservation Bioacoustics, Cornell University) for his generous help whenever I approached him. He introduced me to the teams from the University of St Andrews. With the help of Dr. Danielle Harris and Prof Len Thomas from St Andrews, I designed a systematic wolf howling survey for the population estimation trial. Only two months of fieldwork were left to figure out whether the protocol works. I thought I would finish my Ph.D. by the end of 2020. Who knew that the world would be different than we all could ever imagine? I was stuck in the field in 2020 due to covid lockdown. It was a room in grassland with a metal roof and frequent power cuts. My fellowship also stopped as our project was till April 2020 only. I could only cover 25 howling survey points out of 100 systematic howl survey targets. Now my project and permission both got over. And life took to a halt. The first month departed just to understand the severity of the situation. The person who stood with me was Ms. Soma Sarkar. She helped me maintain my calm and make the most of the situation. My abstract was accepted for the International Statistical Ecology Conference, which became virtual due to Covid Outbreak. I decided to proceed with the data I had and focused only on the conference presentation. This helped me to start working on my final chapters. I managed to do a very initial level analysis and presented that at the conference. The chair of the session, Dr. Tiago A Marques, found my study interesting. Prof Thomas set a meeting with some excellent dignitaries like Prof David Borchers and Dr. Tiago A Marques. I also met Ben Stevenson at this conference, who was working on Acoustics Spatial Capture-Recapture Model. I understood that I could still do a lot many things with the data I had. The discussions from the conference gave new impetus to my

Ph.D. journey. As new avenues were opening in my Ph.D. journey, the world was re-opening with new hope after Covid. I was still not sure when and how I would be able to escape from the field station. I will give the entire credit to Zehidul (Mr. Zehidul Hussain), my roommate, who took the initiative to get me back to the headquarters at Dehradun. Soon after reaching Dehradun, with the help of Tiago and his students, I refined the analysis of my third chapter, i.e. howling behaviour, and presented it at the 57th Annual Conference of the Animal Behavior Society (Virtual).

Within two months of reaching Dehradun, another covid outbreak happened. But I was prepared this time with an action plan. I finished two manuscripts (3rd and 4th Ph.D. Chapters) within the next couple of months. I was privileged to have support from co-Author Holly during that time. She helped me a lot in finalising these papers. We published the article in March 2021. Still, work remained for my thesis's final chapter. I got great support from Ben (Dr. Ben Stevenson) and used his package for the population estimation of wolves. While all was going well, my laptop suddenly crashed. I gave it to the service center as another lockdown put our lives to a halt. Although the data was safe due to routine backups, I did not have a laptop to do any work. I could not even order one laptop as only purchasing essential services was allowed. After nearly one and a half months, laptops were available for online shopping. Finally, I got one and finished the analysis of the final chapters, and presented my pre-thesis synopsis in Wildlife Week on October 5, 2021. I finished writing my thesis in the first week of November 2021.

After many arguments and professional and personal struggles, I submitted my thesis with further refinement on February 15, 2022, to the University. I got immense personal and professional support from my Supervisor, Dr. Bilal Habib, Dr. Bitapi Sinha (Research Co-Ordinator), and Dr. V. P. Uniyal (Nodal Officer, External Affiliation) throughout the journey. I was pursuing my dream, but it was an outstanding level of patience for my family and my would-be in-laws' family. Even during the worst financial and personal struggles, none of them left my hand, especially my partner, Ms. Soma Sarkar, who witnessed all my battles and successes with me.

My Ph.D. was not just my own journey; it was a collaboration.

Executive Summary

The Indian wolf is a Schedule I species in the Wildlife Protection Act 1972. It is now considered an *Evolutionary Significant Unit* (A adaptive variation significantly important for conservation) (Hennelly et al., 2021). Since they survive predominantly in a human-dominated landscape (Habib et al., 2021; Habib & Kumar, 2007), they face immense survival threats due to habitat degradation and man-animal conflict (Agarwala et al., 2010). Their population status has remained unassessed over the years due to difficulties associated with the population estimation of this visually cryptic long-ranging species (Cozzi et al., 2021). A few studies have suggested that around 1000 to 2000 (Sillero-Zubiri et al., 2004) wolves are left in India, but those are rough estimates without statistical support. Therefore, a non-invasive statistical tool is required to estimate this visually cryptic species. Since the howling survey is considered the most efficient monitoring tool for this visually cryptic species (Harrington & Mech, 1982), my study aimed to standardise a statistical tool to estimate the population of Indian wolves based on their howl. I have started my work with a single point of reference on Indian wolf vocalisation – a comparative study of Indian wolf howls with a few other subspecies (Hennelly et al., 2017). I began the study with howling survey responses and opportunistic recordings from captive and nine free-ranging packs of Indian wolves. Different harmonic call types were characterised using an unsupervised statistical tool and defined to generate baseline information about the vocal characteristics of the Indian wolf. Through unsupervised clustering, I found four distinct vocalisations using 270 recorded calls (Average Silhouette width Si = 0.598), which include howls and howl-barks (N = 238), whimper (N = 2), social squeak (N = 28), and whine (N = 2). Indian wolf howls have an average mean fundamental frequency of 422 Hz (±126), similar to other wolf subspecies. The whimper showed the highest frequency modulation (37.296±4.601) and the highest mean fundamental frequency (1708±524 Hz) compared to other call types. Less information is available on the third vocalisation type, i.e. 'Social squeak' or 'talking' (Mean fundamental frequency = 461±83 Hz), which is highly variable (coefficient of frequency variation = 18.778±3.587). Lastly, I identified the whine, which had a mean fundamental frequency of 906Hz (±242) and was similar to the Italian wolf (979±109 Hz). The study highlighted how 'social squeak' can be misidentified with the howl. They can be differentiated through their frequency modulation and duration. Social squeaks ($\overline{x}$ = 3.87s) are generally shorter than howl ($\overline{x}$ = 5.214s). My study on the characterisation of the harmonic vocal repertoire provides a first step in understanding the function and contextual use of vocalisations in the Indian wolf.

Studies over the years found that wolf howls contain individual-specific information (Fentress, 1967; Root-Gutteridge et al., 2014b, 2014a; Tooze et al., 1990). But identifying the unknown individual from their howls had remained challenging over the years, without which howl could not be used in Capture-Mark-Recapture studies (Marques et al., 2013; Stevenson et al., 2015). By understanding the importance of howl identification to an individual in population estimation, I trained a supervised model using known howls to identify howls to individuals. I verified the model with a set of unknown howls (unknown to the model). In this supervised classification, I achieved 97.9% accuracy in identifying known howls (trained dataset) and 75% accuracy in identifying unknown howls (test dataset). For the first time, the unknown wolf howls were classified successfully. Although the achievement is very significant in wolf vocalisation research, further accuracy is required for using them in the population estimation model. Training the model with more howls and verifying them with a different set of test data might increase its reliability. For these, a continuous recording of captive individuals and recordings from free-ranging collared wolves for an extended period is essential.

The howling behaviour of Indian wolves has never been studied. Therefore, understanding the howling behaviour of the Indian wolf was the key to designing a howl survey methodology for population estimation. I studied the howling behaviour of collared and non-collared free-ranging wolves through the response pattern of the active howl survey. I found a disparity in their howl response - based on the distance to villages. In the low disturbed East-Maharashtra (EM), wolves mostly avoid responding to howling surveys (HS) if done within 1200 meters of villages [Response Rate(RR)=0.03±0.021], but they do respond once it is done far from villages (>1200m)[RR=0.226±0.075]. In high human dense West-Maharashtra (WM), wolves showed high RR within 1200 meters from the villages (RR=0.148±0.031). But the RR within 500 meters from villages is less as howling near villages might owe to easy detection. The collared wolf data showed significantly high RR (0.635±0.067) in their home-range core but low RR if the core area is close to a village. Therefore howling too close to the village is disadvantageous, although their tolerance for responding to HS has increased in the human-dominated landscape. The extent of the village may increase further with development, which will leave fewer areas for the wolf to defend territory with a long-range howl. The wolves might behaviourally adapt to a human-modified landscape by reducing their howling intensity.

Adaptation in a fragmented habitat may save the wolves from extinction, but the repercussions of the fundamental behavioural alteration might adversely impact wolf behaviour and the ecological cascade. Whereas ecologists are mainly concerned with the extinction of species, the study highlights the vulnerability of fundamental behaviour of a keystone species attributed to human-induced contemporary evolution.

Based on the vocalisation behaviour, I found that a howl survey should be done during their pre-denning season (November-December). Additionally, wind speed is low during this period. The best grid size for a systematic grid howl sampling is 1.7×1.7 km^2. A 30watt speaker should be used for an active howl survey with 3-5 trials. This study provides the crucial guideline for a howling survey in Indian conditions. Based on these criteria, a howl survey was designed for four districts of Maharashtra. Maximum Entropy Probably Distribution (Maxent) was used for delineating the potential wolf habitats, and 12250 km^2 effective wolf habitat was found. A newly triple observer-based howl survey method was introduced, I obtained a relatively high howl response (seven out of twenty-five howl surveys) in randomly selected grids. I used 'redetection' in different points in space instead of using individual 'recapture' with time. Through my pilot study, I found Indian wolf density is 3.65 individuals/100 km^2 with a lower limit of 1.67 to an upper limit of 5.63 (95% CI). Although I do not have data on the population density of Indian wolves to compare, the data and its error range are comparable with the population density of Iberian wolves, i.e., 2.55 wolves/100 km2 (95% CI = 1.87–3.51) estimated by DNA (scat) sampling by López-Bao et al. (2018). The standard error might decrease further with an increase in sampling effort through the active howl survey. This methodology can be a guideline for using the active howling survey in the population estimation of wolves globally.

Since wolf howls also possess individual information, incorporating this information in the future will help reduce the bias and heterogeneity in the population estimation model. Incorporating individual identification in the population estimation model will help generate additional details such as animal survival and home range. Regular population monitoring will help conserve and save this cryptic species before its population falls below a recovery level. Therefore, the study is a stepping stone towards using bioacoustics to estimate animal density and play a significant role in global wolf conservation.

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CHAPTER 1 Introduction

1.1 Grey Wolf and their Ecological Role

Grey wolves (*Canis lupus*) are the largest member of the Canidae family and play the role of '*Apex Predator*' by shaping the structure and function of the ecosystem (Ripple & Beschta, 2012; Roemer et al., 2009). They are highly adapted to a wide range of habitats worldwide (Boitani et al., 2018). Wolves in Northern America largely depend on large ungulates to medium-sized mammals, whereas in Europe, their predominant diet is medium-sized wild ungulates (Newsome et al., 2016). However, in Asia, the unavailability of wild prey alters their diet pattern toward domestic prey and human-subsidised food (Newsome et al., 2016; Petroelje et al., 2019). Anthropogenic food subsidies shift their ecological role, and dependency on livestock or carcases increases the chance of human-wildlife conflict, especially in human-modified landscapes (Ciucci et al., 2020; Kuijper et al., 2016). One wolf subspecies that survived in the human-modified landscape is Indian Wolf (*Canis lupus pallipes*) (Habib, 2007; Jhala & Giles, 1991; Singh & Kumara, 2006). The Indian wolf is one of the smallest subspecies of the Grey wolf and has the oldest lineage of the present-day grey wolf population (Hennelly et al., 2021). A large portion of their diet consists of domestic livestock or village food subsidies (Habib, 2007; Jethva & Jhala, 2004b). Besides this, they also depend on smaller to medium size wild prey such as blackbuck (*Antilope cervicapra*), chinkara (*Gazella bennettii*), wild pig (*Sus scrofa cristatus*) and others (Habib, 2007; Jethva & Jhala, 2004a; Kumar & Rahmani, 2000, 2008). By predation on ungulates, wolves regulate their population and impose a 'landscape of fear' among domestic and wild prey (Ordiz et al., 2013; Ripple & Beschta, 2012). The impact of the wolf has a significant role in the ecosystem recovery and passive restoration of the tropical cascade (Ripple & Beschta, 2012).

1.2 Social Structure and Behaviour

Wolves are highly social animals and exist in packs (Mech & Boitani, 2003). Their pack consists of 'breeding parents' and offspring from different years (Mech & Boitani, 2003; Schenkel, 1947). The 'Breeding male' and 'breeding female' lead the pack equally and are socially dominant (Peterson et al., 2002; Schenkel, 1947). The 'sub-ordinate breeding female also leads the pack during movement (Peterson et al., 2002). The pups are taken care of mostly by the 'breeding female' with assistance from subordinates within the pack (Creel, 2005; Schenkel, 1947). Instead of spending time in the den, 'breeding males' invest more time leading the foraging and food provisioning during the pup-raising season (Mech, 1999). During this time, 'breeding males' also move around the vast areas to maintain the territories (Alfredéen, 2006; Tsunoda et al., 2008). When males or other individuals are away from the den or pack, their long-distance vocalisation (howl) plays a crucial role in locating the pack and conveying alarm in extensive areas (Mazzini et al., 2013; Watson et al., 2018). Subadults leave their pack to disperse in search of a possible mate (Kochetkov, 2015; Kojola et al., 2009). During this solitary dispersal phase, howl plays a critical role in avoiding physical conflict with native packs of the area and finding a possible mate in an extensive landscape (Harrington & Mech, 1978b; Watson et al., 2018). Besides howls, wolves communicate through visual cues mediated by different body postures, scents, and vocal repertoires (Harrington et al., 2003; Harrington & Mech, 1978b; Scott, 1967).

1.3 Howl and other vocalisations

Wolves use various types of vocal repertoire to communicate. Their vocal repertoire consists of either harmonic calls (Laryngeal sound involves with vocal fold) or noisy calls (tracheal sound through resonating vocal tract) (Frank, 1987; Harrington & Mech, 1978b; Joslin, 1966). The prime example of a harmonic vocal call is the howl (Frank, 1987; Harrington & Mech, 1978b). Due to its low frequency and high energy, a howl can travel up to 6 km (depending upon weather and terrain) and helps wolves maintain vast territories alongside communicating with pack members over distances (Harrington & Mech, 1978b, 1983; Joslin, 1967; Mazzini et al., 2013; O'Gara et al., 2020). The whimper, yelp and whine are short-distance harmonic vocalisations predominantly used as friendly gestures (Harrington et al., 2003; Harrington & Mech, 1978b; Joslin, 1966). Whimper calls are used for greeting, whereas whine and yelp represent submissive behaviour (Harrington & Mech, 1978b; Joslin, 1966). A Wolf generally shows aggression through different noisy vocal repertoires such as snarl, woof, bark and growl (Harrington & Mech, 1978b; Schassburger, 1993). Each repertoire represents a different degree of aggression. They also mix different vocal repertoires (both harmonics and noisy calls) to transmit complex vocal signals, such as wolves using howl-bark to show aggression to the enemy and sending an alarm to pack members (Harrington & Mech, 1978b).

1.4 Distribution and Population status

Wolves were once among the highest distributed and highly adapted land mammals, residing in almost 16 different habitat types (38 subspecies) around the globe (Boitani, 2018; Mech et al., 2010). In the past century, wolves lost two-thirds of their habitat and were extirpated from Mexico, most of the USA, Western Europe and Japan (Boitani, 2018; Mech et al., 2010; Mech & Boitani, 2003). Modern wolf populations range in the remote areas of Northern USA and Canada, Europe and Asia (Mech & Boitani, 2004). Indian wolves have a wide distribution range in the subcontinent - latitudinally from Rajasthan to Karnataka and Longitudinally from Gujrat to West Bengal (Gubbi et al., 2020; Jhala & Giles, 1991; Saren et al., 2019; L. K. Sharma et al., 2019). Indian wolves are primarily found in non-protected areas. They inhabit mainly the village outskirts and frequently contact humans (Habib & Kumar, 2007; Jhala & Giles, 1991; L. K. Sharma et al., 2019; Singh & Kumara, 2006).

Although historically, wolves were the widest distributed terrestrial carnivore around the northern hemisphere, wolves now face extinction threats at subspecies or the local level due to escalating anthropogenic pressures (Berger, 1999; Gómez-Sánchez et al., 2018). The primary threat driving the declining wolf population is habitat fragmentation, harvesting (mainly in the US and Europe) or conflict due to livestock depredation (Mech et al., 2016; Rich et al., 2012). Besides the threat and population decline, the actual status of the world wolf population is yet unknown due to the unavailability of a standardised non-invasive population estimation method (Garland et al., 2020; López-Bao et al., 2018; Papin et al., 2018). Although the population of wolves in the Indian peninsula is believed to be around 2000-3000 (Sillero-Zubiri et al., 2004), those figures are approximate projections.

1.5 Challenge associated with wolf pack census

The population size of any species determines its conservation status and earnestness (Reed, 2005). The regular monitoring of keystone species helps assess the whole ecosystem over time (Johnson et al., 2017). As a keystone species, the wolf balances the entire ecological pyramid by controlling the prey population (Beschta & Ripple, 2012; Hale & Koprowski, 2018; Ripple & Beschta, 2012). However, the widely used animal census methods, such as transect sampling and camera trapping, fall short of estimating the wolf population due to their extensive home range and identification difficulties (Garland et al., 2020; Papin et al., 2018). Likewise, DNA-based non-invasive methods may result in biased population estimation due to individual wolves' differential scent marking patterns (López-Bao et al., 2018). Although the occupancy model has been used to determine their habitats, the critical challenge in the habitat occupancy model is their false positive data (Miller et al., 2013; Rich et al., 2013). Applying an occupancy-based population census model may result in erroneous estimation in the human-dominated landscape with dog pug marks and scats. Therefore, a standardised wolf census protocol will enable a reliable estimate of the global wolf population.

1.6 Wolf pack census using howling response

Wolves are visually cryptic, but they advertise their presence and defend their territories through howling (Harrington & Mech, 1978b, 1978a; Joslin, 1967). As howl is a territorial call, wolves respond to howls from other individuals or pre-recorded calls to defend the territory (Harrington, 1987; Joslin, 1967). The howl response can be heard from as far as 6 km from the origin, making a howling survey one of the efficient non-invasive tools for wolf pack census (Harrington & Mech, 1982). Besides locating the pack, a howling survey is an efficient tool for detecting wolf homesites and rendezvous sites (Fuller & Sampson, 1988; Gable et al., 2018; Iliopoulos et al., 2014). Since wolves mostly live in packs, they respond together as a pack to the howling survey (Palacios et al., 2016; Passilongo et al., 2017). This group vocalisation is known as chorus howl. The number of individuals present in a chorus can be calculated through the spectral view or acoustics index of the chorus howl (Papin et al., 2019; Passilongo et al., 2015). Wolf pups usually vocalise in higher frequencies; the presence of pups in a chorus song can also be detected through the energy distribution model (Palacios et al., 2016). Therefore, the wolf pack census data through the howling survey provide various crucial information such as wolf homesites, pack composition and the reproductive success of the pack (Iliopoulos et al., 2014; Llaneza et al., 2014; Palacios et al., 2016; Passilongo et al., 2015).

1.7 Howl as a tool to identify individual

One of the initial scientific papers on howls was published in 1967 by John Theberge and J. Falls (1967). They studied 700 howls from three timber wolves and divided the fundamental frequency of each howl into three parts, i.e. starting part, middle part and end part. They studied variation in harmonics among the individuals along with the variation within the individuals. They also found a few unique features separating the howls of two individuals. Though they did not aim to identify individuals by using howl, this study indicated the ability of a wolf to distinguish the howl of the different individuals. In 1990 a paper was published by Z. Tooze and his associates about the distinct vocalisation of timber wolves (1990). They summarised 14 variables by which wolves can be identified at the individual level by using the fundamental frequency of howl. The most significant finding of this study was that they distinguished known individuals with 82% accuracy. In 2012, scientists found that wolf howl structures significantly differ among packs (Zaccaroni et al., 2012). However, they could not distinguish different packs from the howl signature. In 2013, Root-Gutteridge et al. successfully identified 89 solo howls with 100% accuracy. After including histogram derived PCA and Amplitudes together, they reached 100% accuracy in identifying solo howls and 97% accuracy for chorus howls (112 chorus howls of 10 individuals) (Root-Gutteridge et al., 2014a, 2014b). These papers did not deal with Identifying unknown individuals from howls; the methodology for determining the number of individuals from an unknown set of howls is not available to date. Identifying unknown howls to individuals would open a new horizon for mark-capture-recapture-based population estimation methods for wolves.

1.8 Scope of the current study

The Indian wolf is among the oldest lineage of modern wolves, hence considered a Significant Evolutionary Unit (ESU) (Hennelly et al., 2021). Besides its colossal conservation importance, the actual population status is entirely unknown. Although the howling survey can estimate the wolf population, the methodology has yet to be standardised. Moreover, very little information is available on howls and other types of vocalisation of the Indian wolves.

The current study addresses the knowledge gap about Indian wolf vocalisation. Alongside, it focuses on the identification potentiality of wolf howl to an individual level for using them in population estimation through mark-capture-recapture. The study also includes the howling behaviour and responses of the Indian wolf to various ecological and anthropogenic factors. Understanding the howling behaviour is key for designing howl survey methods for wolf census. As howl survey is a technique to detect wolves over long distances, the technique might provide a cost-effective solution for population estimation and a non-invasive monitoring tool for Indian wolves in human-dominated landscapes. Besides the clear idea about the population status of Indian wolf, the standardised howling survey method can reveal various pieces of information such as social behaviour (Biben, 1983; Faragó et al., 2014; Joslin, 1966), ecology (McIntyre et al., 2017), breeding success through detection of pups (Palacios et al., 2016), and even evolutionary history (Chen & Wiens, 2020; Hennelly et al., 2017; Kershenbaum et al., 2016). This standardised protocol for wolf pack census can also be applicable for studying other wolf subspecies. Therefore, the study would significantly contribute to global wolf conservation.

1.9 The objectives of the study

Wolves play a critical role in ecosystem structure and landscape sustainability. Indian wolves are the keystone grassland species and are considered an ESU. Besides their importance in the ecosystem, the population status of Indian wolves is entirely unknown because of the unavailability of the standardised protocol for the population estimation of wolves. Previous studies have posited that wolves' howls can be an effective tool for wolf pack census due to its long detectability. The principal aim of this study is to *"Understanding Wolf Howls & Their Application in Individual Identification & Population Estimation"*. Since there is a significant knowledge gap about the howls and howl behaviour of Indian wolves, my study will characterise the howls and the howling behaviour of Indian wolves. Understanding the howl and howl behaviour of the Indian wolf will help design an effective non-invasive tool for wolf pack census through the howling survey. This study covers the potentiality of identifying wolves from howl to individual for effectively implementing mark-capture recapture in the population model. This study was designed with the following objectives to verify the feasibility of wolf pack census and population estimation using a howling survey:-

1. Characterising Indian wolf Howls
2. Howl response variation in Indian wolves and playback response
3. Howling as a tool to identify individuals
4. Possible method for population estimation of wolves using howls

1.10 Study area

The study was conducted on captive individuals of Jaipur Zoo and free-ranging wild wolves of Maharashtra, India.

Jaipur Zoo is situated at the centre of Jaipur City, Rajasthan, India. All the wolves (n=10) in Jaipur zoo were offspring of captive-bred individuals except one adult male recently captured from a wild population of Rajasthan.

The data on free-ranging wild wolves were collected from six districts of Maharashtra: Pune, Ahmednagar, Solapur, Osmanabad, Nagpur and Gondia. Pune, Ahmednagar, Solapur and Osmanabad fall under the semi-arid drought-prone area of the Deccan peninsula

Biogeographic Zone (Zone 6) (Rodgers & Panwar, 1988). In my sampling areas, the dominant habitat type was Deccan thorn scrub forests (Reddy et al., 2015). The terrain gently undulates with mild slopes and flat-topped hillocks with intermittent shallow valleys, forming the primary drainage channels. Grassland is distributed in fragmented patches, creating a mosaic of grazing land, agricultural land and human settlements.

Nagpur and Gondia districts come under the central Deccan Plateau with Tropical dry deciduous broadleaf forests (Reddy et al., 2015; Rodgers & Panwar, 1988). Due to moderate to high rainfall, vegetation is dense in most areas. My sampling areas were mainly packed with open forest and modest density forest. The terrain is generally flat. Nagpur division is surrounded by Many National parks and Sanctuaries. Wolves are primarily found in the buffer areas of National parks and sanctuary boundaries.

The data was collected in two phases. In the first phase, the howling surveys were conducted on the free-ranging non-collared wolves from December 2015 to December 2019 in the Deccan Peninsula and Vidarbha. In the second phase, the data was collected from January 2018 to July 2019 in the Deccan Peninsula on five radio-collared wolves.

1.11 Chapter organisation

Chapter one introduces the basic ecology and ecological role of the Indian wolf. Through the literature review, I portrayed the importance of studying Indian wolf vocalisation and how a howl survey can be an effective non-invasive tool for monitoring and population estimation of the species.

Chapter two primarily focuses on characterising the Indian wolf howl. Besides howls, this chapter also describes other harmonic calls found in the howls of Indian wolves. Through a statistical model, different call types were characterised and defined to generate baseline information about the vocal characteristics of the Indian wolf.

Chapter three covers the potentiality of identifying wolf howls to individuals. Previous studies have demonstrated that howls have individuality information, and known wolves can be differentiated based on their howls. In this, I attempted to identify unknown wolves from their howls.

Chapter four demonstrates the howling behaviour of Indian wolves based on howling survey responses. This chapter describes some critical determining factors for wolf response rate. The study is a milestone for understanding the howling behaviour of the Indian wolf, which would provide crucial information for a successful howling survey design for population census. The Chapter also highlighted some critical alterations in the vocalisation behaviour of the Indian wolf to Anthropocene in India, which is a significant conservation concern.

Chapter five describes a new technique for population estimation of Indian wolves through a howling survey. The chapters demonstrate the sampling area design alongside the howling survey methodology. The population estimation is done through a howling survey in four districts of Maharashtra, also included in this chapter.

CHAPTER 2 Characterising the harmonic vocal repertoire of the Indian wolf (*Canis lupus pallipes*)

This chapter has been published

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2.1 Introduction

Vocalisation plays a critical role in social animals for conveying information on foraging, reproductive, and social behaviours (Garber et al., 2009; Gazzola et al., 2002; Harrington & Mech, 1982; Kingston et al., 2001; Nawroth et al., 2016; Scott, 1967; Theberge & Falls, 1967). Characterising the vocal repertoire of a species provides a base for understanding the behavioural significance of different vocalisations and studying how vocal communication varies across populations, subspecies, and taxa (Kershenbaum et al., 2016; Tembrock, 1963; Wilkins et al., 2013).

The wolf (*Canis lupus*) is a social mammal and uses a variety of vocalisations for communication. Being present throughout Eurasia and North America, the wolf is one of the most widely distributed land mammals and occupies a wide range of different habitat types (Mech et al., 2010). While there has been much research on wolves in North America and Europe, much less has been done on the wolves of Asia. For the grey wolf, most of the mitochondrial diversity is centred in southern and central Asia, where two independent and phylogenetically basal maternal lineages –the Tibetan and Indian wolf– are found (Ersmark et al., 2016; D. K. Sharma et al., 2004; Werhahn et al., 2017). The Tibetan and Indian wolf maternal lineages are estimated to have diverged around 700,000, and 300,000 years ago, respectively (Aggarwal et al., 2007; D. K. Sharma et al., 2004; Shrotriya et al., 2012). Despite its phylogenetic position as one of the oldest maternal lineages and among the smallest subspecies (Aggarwal et al., 2007), relatively little is known about Indian wolf ecology and behaviour compared to other wolf subspecies. Studying the vocalisations of the Indian wolf can offer a greater understanding of the behavioural function of different vocal signals in Indian wolves and, more broadly, the variation in vocalisation and associated behaviour across subspecies and taxa within the *Canis* clade.

The best-known wolf vocalisation – the howl – is a long-range harmonic call used for territorial advertising and social cohesion (Harrington, 1987; Harrington & Mech, 1978a; Schassburger, 1993; Theberge & Falls, 1967). Wolf howl acoustic structure has been shown to vary across individuals (Palacios et al., 2007; Root-Gutteridge et al., 2014b, 2014a; Theberge & Falls, 1967; Tooze et al., 1990; Watson et al., 2018), groups (Zaccaroni et al., 2012), and subspecies (Hennelly et al., 2017; Kershenbaum et al., 2016). Among the *Canis* clade, smaller species generally have howls that end in a sharp drop in frequency and a greater diversity of howl type usages (Kershenbaum et al., 2016). Previous research has shown that Indian wolf howls generally have a higher mean fundamental frequency compared to other wolf subspecies, which may be attributed to its smaller body size (Hennelly et al., 2017). Using a larger set of howls that are statistically classified by their acoustic features can provide a more robust description of the characteristics and diversity of Indian wolf howl types.

Along with the howl, wolves also communicate using seven to twelve other harmonic calls (Cohen & Fox, 1976; Coscia et al., 1991; McCarley, 1978). Harmonic calls are produced by the vibration of vocal folds in the larynx, which results in a series of multiple integral frequencies of the fundamental frequency (Faragó et al., 2014). Many of these other harmonic vocalisations are short-ranged, and due to difficulties in recording these calls, remain less studied compared to the wolf howl (Mech, 1981). These short-ranged calls are essential for communicating passive or aggressive behaviour among social canids (Feddersen-Petersen, 2000; Mech, 1981; Mech & Boitani, 2010). Grey wolves also use non-pitched or noisy calls, which are produced by the acoustic resonance of the vocal tract (Fentress, 1967; Joslin, 1966; Mech & Boitani, 2003; Schassburger, 1993). Instead of a specific frequency band, noisy calls possess concentrated acoustic energy around a particular frequency range. Therefore noisy calls do not have a clear pitch or distinct frequency band in their spectrograms (Faragó et al., 2014).

The whimper, whine and yelp are various harmonic calls for communicating passive and friendly behaviour among wolves (Mech & Boitani, 2010; Schassburger, 1993), whereas noisy calls such as growl and bark indicate varying levels of aggression (Mech & Boitani, 2010; Schassburger, 1993). The whimper, and whine vocalisations are similar to a crying sound with the whimper having a comparatively shorter duration than whine (Mech & Boitani, 2003; Schassburger, 1993). The whine vocalisation is mostly used for submissive behaviour, whereas the whimper is primarily used for greeting (Schassburger, 1993). The yelp is a short and sharp cry vocalisation that is associated with submissive behaviour involving body contacts (Mech & Boitani, 2003; Schassburger, 1993). To communicate different levels of aggression behaviours, wolves use noisy calls, which consist of the growl, woof, and bark. Growl is a non-harmonic sound to show dominance in any interaction, whereas the woof vocalisation is a non-harmonic sound cue used by adults for their pups (Mech & Boitani, 2003; Nikol'skij & Frommol't, 1989; Schassburger, 1993). The bark is a short, low pitched sound with rapid frequency modulation and is used during aggressive defence (Coscia et al., 1991; Nikol'skij & Frommol't, 1989), such as defending pups or defending a food resource (Mech, 1966; Scott, 1967). Wolves also express communication through mixed vocalisation either by 'successive emission' or by 'superimposition' of two or more sound types (Cohen & Fox, 1976). A recent study on the Italian wolf (*Canis lupus italicus*) suggests six other types of calls may combine with howls to make a complex chorus vocalisation (Passilongo et al., 2017).

This study investigates the acoustic structure of harmonic vocalisations of Indian wolves and classifies these harmonic vocalisations using a statistical approach. We accumulated the vocalisation data from free-ranging and captive Indian wolves, which will be the first study to evaluate different types of vocalisations of this wolf subspecies. Using multivariate analyses, we describe and classify different harmonic calls to develop a vocal repertoire of the Indian wolf.

2.2 *Materials and methods*

2.2.1 *Study Species*

The Indian wolf (*Canis lupus pallipes*) is among the smallest wolf subspecies with an average body weight of 20.75 kg (Habib, 2007). Indian wolves are mostly found in grasslands and the edges of dense tropical deciduous forest on the Indian subcontinent (Habib, 2007; Habib & Kumar, 2007; Jethva & Jhala, 2004b; Morin et al., 2016; Singh & Kumara, 2006). The average home range of a pack varies from 180-250 km^2 (Habib, 2007). We recorded vocalisations from nine packs of free-ranging wolves and ten captive wolves from Jaipur Zoo. For captive wolves, we collected vocalisation data from 10 wolves: two adult pairs and six subadults. One adult male was recently captured from the wild near the city of Jaipur, Rajasthan, India. The rest of the Indian wolves are descendants of captive breeders at Jaipur Zoo.

2.2.2 *Study Sites*

This study was conducted in the state of Maharashtra (**Figure 1**) and Jaipur Zoo of Jaipur, Rajasthan, India. The study site in Maharashtra was located on the central Deccan Plateau (Rodgers & Panwar, 1988), which consists of the overlapping habitat of tropical dry deciduous forest, grassland, savanna (Western part) and tropical moist deciduous forest (Eastern part) (Reddy et al., 2015).

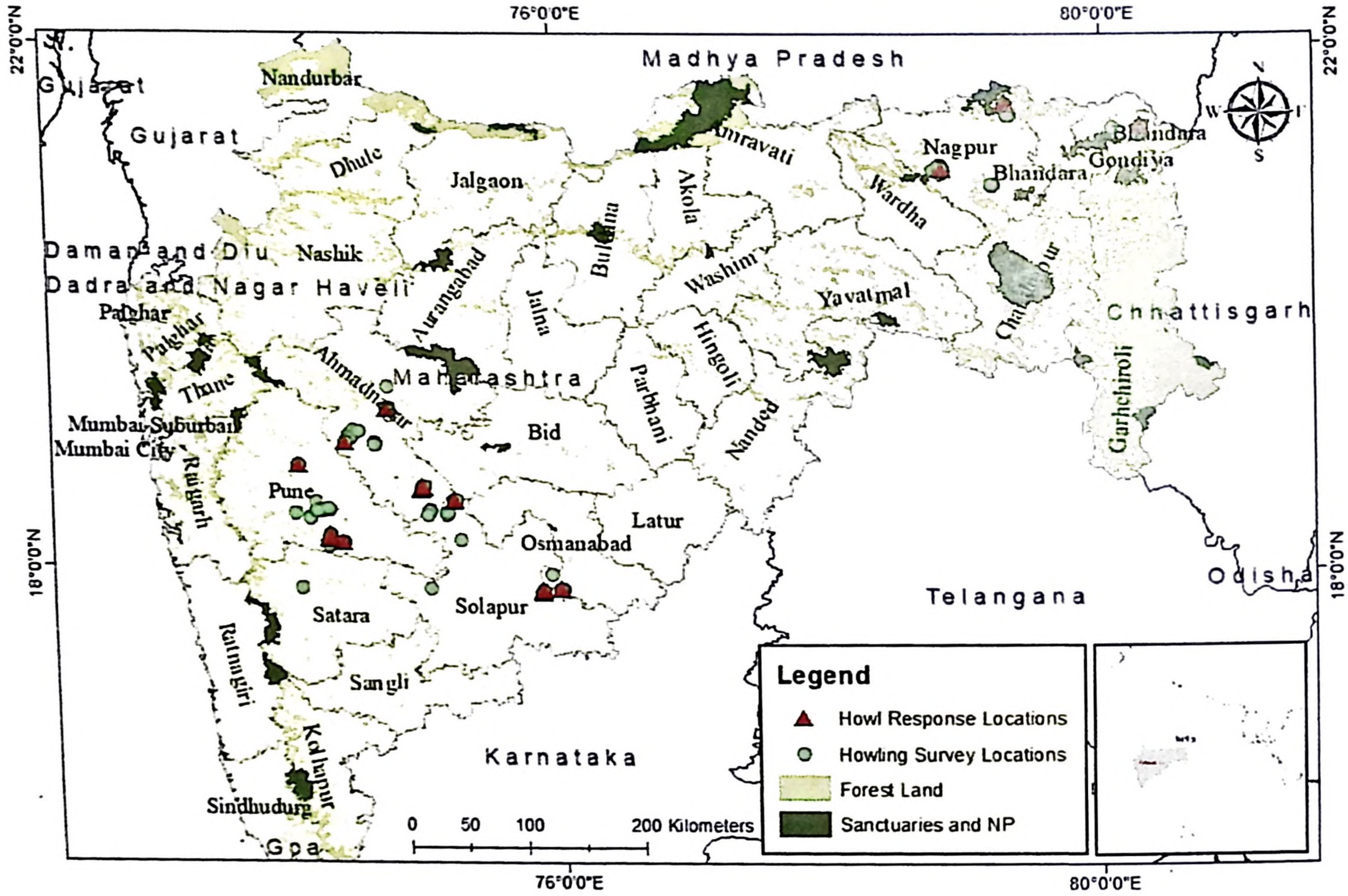

Figure 1. Map of survey sites of the free-ranging wolves. *Green round bullets indicate the survey locations and Red triangular bullets represent the howling response sites.*

2.2.3 *Data Collection*

Vocalisations of free-ranging wolves were recorded through acoustics survey from November 2015 to June 2016. The majority of the long-distance vocalisation recordings were collected through howling surveys to elicit howl behaviour. Opportunistically, spontaneous howls were also recorded. For other types of vocalisation data, we relied on opportunistic recordings from free-ranging wolves and captive wolves. Howl surveys were performed during early morning and evening hours using pre-recorded howls that were previously recorded from the Jaipur Zoo Indian wolves. Each howling session consisted of five trials with three minute long intervals (Harrington & Mech, 1982). A 50-second-long pre-recorded sequence of a solo howl was played three times using JBL Xtreme speakers (Harman International Industries, 2014) in order of increasing volume (Harrington & Mech, 1982). The session was followed by two 50-second-long chorus howls. In the case of a howling response, the session was terminated and repeated after 15 to 20 minutes (Harrington & Mech, 1982). Responses were recorded using Blue Yeti Pro Microphone (Blue Microphone, 2011) attached with Zoom H4N Handheld Audio Recorder (Zoom Corporation, 2009) at a sampling rate of 44.1KHz on 16-bit depth with 80 Hz noise filter. Along with howl surveys in the field, opportunistic recording sessions were conducted near wild Indian wolf den sites and rendezvous sites. In addition to howl surveys at Jaipur Zoo, vocalisations of captive wolves were recorded by installing microphones in the front of cages during closing hours (6:30 pm-7: 30 am).

The study on captive wolves in zoos was done with the permission of the Director of Jaipur Zoo and the Forest Department of Rajasthan, India [Letter no- 3(04)-II/CCFWL/2013/4586-87; Dated 30th Oct 2015]. The survey of free-ranging wolves of Maharashtra was performed with the consent of the Principal Chief Conservator of Maharashtra Forest Department [Letter no- 22(8)/WL/CR-947(14-15)/1052/2015-16; Dated-6th Aug 2015]. No animal was harmed during the study, and the standard non-invasive protocol of howling survey was maintained.

2.2.4 Feature Extraction

We focused our analysis on harmonic vocalisations and excluded noisy calls since they do not possess a clear spectral band. Spectrograms of each vocalisation were generated through the Raven Pro 1.5 software (Bioacoustics Research Program, 2014) using the *Discrete Fourier Transform* (DFT) algorithm. The discrete Fourier function transforms the same length sequence of equally spaced sample points (N, where N is a prime number) with circular convolution being implemented on the points (Rader, 1968). *Hann windows* were used at the rate of 1800 samples on 35.2 Hz 3dB filter. A total of 270 spectrograms were selected for further analysis based on clarity (i.e. clearer spectrogram with low noise and without external sound overlap). Web plot digitiser v3 (Rohatgi, 2017) was used for digitising fundamental frequency from the spectral images. This digitised data was obtained at 0.1sec resolution. From this data, eleven acoustic variables (Table 1) were obtained based on their performance from previous studies (Root-Gutteridge et al., 2014b; Tooze et al., 1990).

Table 1. Acoustic variables based on fundamental frequency (f0) that were extracted for this study

Variable Name	Definition of Variable
Min f	The minimum frequency of the fundamental (f_0)
Max f	The maximum frequency of f_0
Range f	Range of f_0; f_0= Max f – Min f
Mean f	Mean frequency of f_0 at 0.1 s interval over the duration
Duration	Duration of Howl measured at f_0; Duration = $t_{end} - t_{start}$
$Abrupt_{0.025}$	Number of abrupt changes in f_0 more than 25Hz at single time step (0.1sec)
$Abrupt_{0.05}$	Number of abrupt changes in f_0 more than 50Hz at single time step (0.1sec)
$Abrupt_{0.1}$	Number of abrupt changes in f_0 more than 100Hz at single time step (0.1sec)
Stdv	Standard Deviation of f_0.
Co-fm	Coefficient of frequency modulation of f_0 = $\Sigma\lvert f(t)-f(t+1)\rvert/(n-1)$ X 100/Mean f_0
Co-fv	Coefficient of frequency variation of f_0= (SD/mean) X 100

2.2.5 *Statistical Analysis*

2.2.5.1 Principal Component Analysis (PCA)

To obtain a smaller set of variables that explain most of the dataset's variation, we used a principal component analysis (PCA), which is an unsupervised statistical approach that extracts linearly uncorrelated variables from a suite of potentially correlated variables (L. I. Smith, 2002). To simplify the interpretation of factors, we performed varimax rotation using Kaiser normalisation (Kaiser, 1959). From our dataset of 270 vocalisations, we used eight scalar variables that are related to spectral structure (Range f, Duration, $Abrupt_{0.025}$, $Abrupt_{0.05}$, $Abrupt_{0.1}$, Stdv, Co-fm, Co-fv) for PCA analysis through the software SPSS (v22). The first principal component (PC1) and second principal component (PC2) were used in the subsequent clustering analyses.

2.2.5.2 Cluster Analysis

To classify the recorded vocalisations from the Indian wolf, we used agglomerative hierarchical clustering through the R package AGglomerative NESting (AGNES)(Maechler et al., 2019). The agglomerative hierarchical clustering algorithm measures the dissimilarity between single and groups of observations using a "bottom-up" approach, thereby constructing clusters (Kaufman & Rousseeuw, 2009). Agglomerative hierarchical clustering was performed using Euclidean distances with PC1 and PC2 from the 270-vocalisation data using eight scalar variables. Subsequently, *silhouette clustering* was combined with AGNES to validate the number of clusters in our vocalisation data. Silhouette clustering measures the similarity of observation within its cluster compared to other clusters (Rousseeuw, 1987). The average silhouette value (0 represents poor fit, 1 depicts the highest fit) describes *the evaluation of clustering validity* (Rousseeuw, 1987). Average Silhouette width (S_i) was calculated for 14 different solutions (2 to 15 clusters). The "solution" that provided the best fit was selected upon the maximum average silhouette value. The dendrogram was plotted using '*Circlize Dendrogram*' in the package '*Dendextend*' in program R (Galili, 2015).

Table 2. The loadings of PC1 and PC2. Communalities are the proportional factors by which the importance of each variable is explained.

Acoustics Parameters	PC1 Loading after varimax rotation	PC2 loading after varimax Rotation	Communalities
Abrupt$_{0.025}$	.858	0	**0.741**
Abrupt$_{0.1}$	.602	.415	**0.535**
Abrupt$_{0.05}$	.825	0	**0.732**
Co-fm	0	.945	**0.898**
Co-fv	.862	0	**0.750**
Duration	0	-.382	**0.231**
Range f	.852	.392	**0.880**
Stdv	.759	.556	**0.885**
% of Variance	48.946	21.692	

2.2.6 Discriminant Function Analysis (DFA)

Discriminant function analysis (DFA) was performed using PC1 and PC2 as an independent variable under the program SPSS (v22) to cross-validate the obtained clusters from AGNES. Predicted clusters that were determined by the maximum silhouette value were then used as a grouping variable to evaluate within-group covariance in DFA analysis. From these clusters, we then used the box plot to show the overall pattern and distribution characteristics of different vocal clusters.

2.3 *Results*

2.3.1 *Principal Component Analysis*

Two principal components (PC1 and PC2) were generated from the eight simple scalar variables through PCA based on Kaiser-Guttman Rule (Eigenvalue >1) (Kaiser, 1991). PC1 and PC2 together explained 70.6% variance. PC1 was based on the variances of six acoustic parameters ($Abrupt_{0.025}$, $Abrupt_{0.1}$, $Abrupt_{0.05}$, Co-fv, Range f, Stdv) whereas PC2 is explained by the variances of five parameters ($Abrupt_{0.1}$, Co-fm, Duration, Range f, Stdv) (**Table 2**)

2.3.2 *Cluster Analysis*

The highest silhouette value ($S_i = 0.598$) was obtained at the 4-group solution in the cluster analysis using PC1 and PC2 from PCA analysis (Figure 2). The average silhouette value was 0.62 for the first cluster (N=238), 0.37 for the second cluster (N=2), 0.38 for the third cluster (N=28) and 0.73 for the fourth cluster (N=2) (Figure 3). The 4 clusters were formed at 3.9 clustering scale through agglomerative hierarchical clustering (Figure 4)

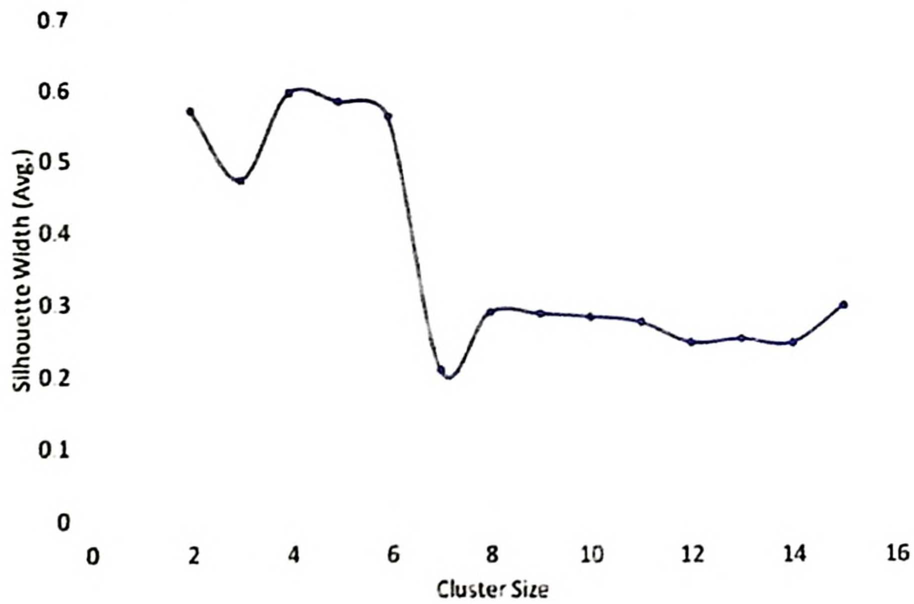

Figure 2. Average silhouette width plotted against 14 different solutions (2-15 cluster). *Average Silhouette width represents the significance level (0 represents poor fit, 1 represents best fit). We obtained maximum silhouette width in 4 cluster solutions, i.e. Si = 0.598*

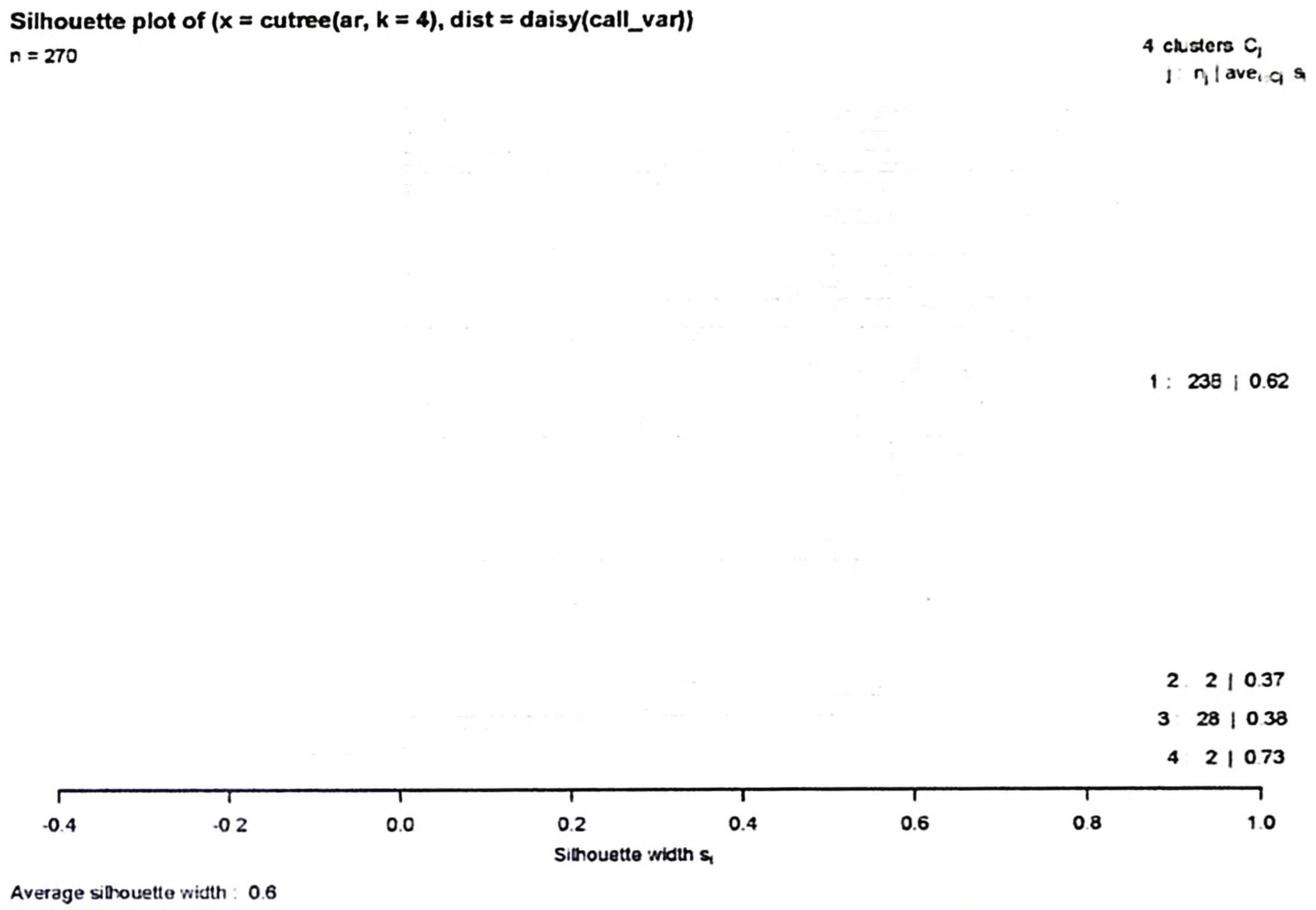

Figure 3. Silhouette plot showing the validation for the consistency among the clusters. *This plot assesses the similarity or difference of each call from its clusters. A negative value indicates the chance of a call to fall under the neighbouring cluster. The average silhouette value of 4 groups are 0.62 (N=238), 0.37 (N=2), 0.38 (N=28), 0.73 (N=2) respectively.*

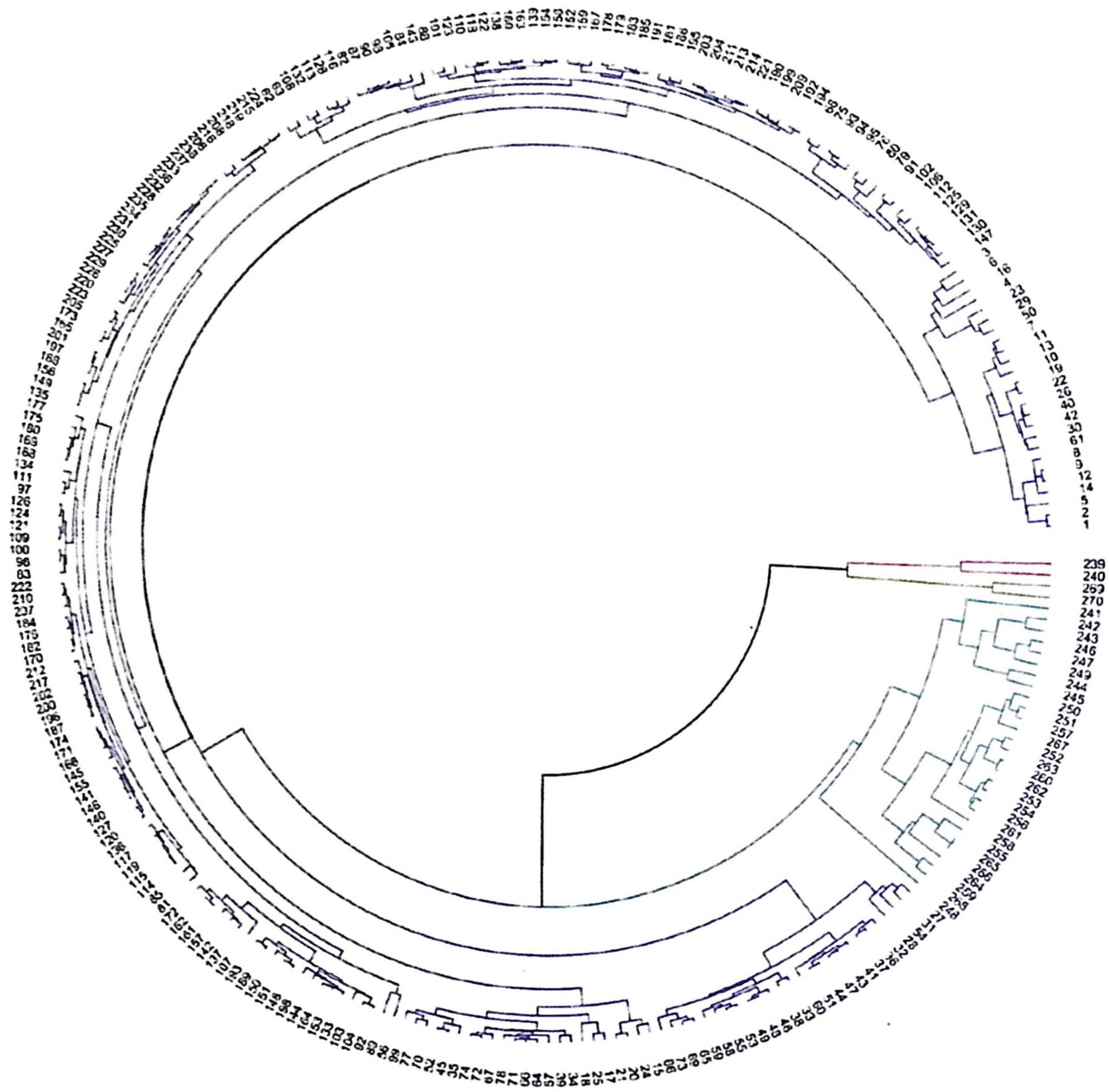

Figure 4. Cluster Diagram obtained from Agglomerative hierarchical clustering using Euclidean Distance as matric function. *Four clusters were formed at 3.9 Clustering scale. Cluster 1 (Howl), Cluster 2 (Whimper), Cluster 3 (Social Squeak) and Cluster 4 (Whine) are denoted by the colours blue, red, green and brown, respectively.*

2.3.3 Discriminant Function Analysis (DFA)

DFA achieved 95.9 % accuracy of vocal group identification using two PCA values (**Table 3**). Each of the four groups has a distinct group centroid. The graphical representation using two discriminant functions (DF1 and DF2) shows that vocal clusters do not overlap (Figure 5).

Table 3. Classification results of Discriminant Function Analysis (DFA). 95.9% of the vocal clusters (estimated from Agglomerative hierarchical clustering) are identified correctly.

		Cluster	Predicted Group Membership				Total
			1	2	3	4	
Predicted in Cluster analysis	Count	1	229	0	8	1	238
		2	0	2	0	0	2
		3	0	0	26	2	28
		4	0	0	0	2	2
	% Correct	1	96.2	.0	3.4	.4	100.0
		2	.0	100.0	.0	.0	100.0
		3	.0	.0	92.9	7.1	100.0
		4	.0	.0	.0	100.0	100.0

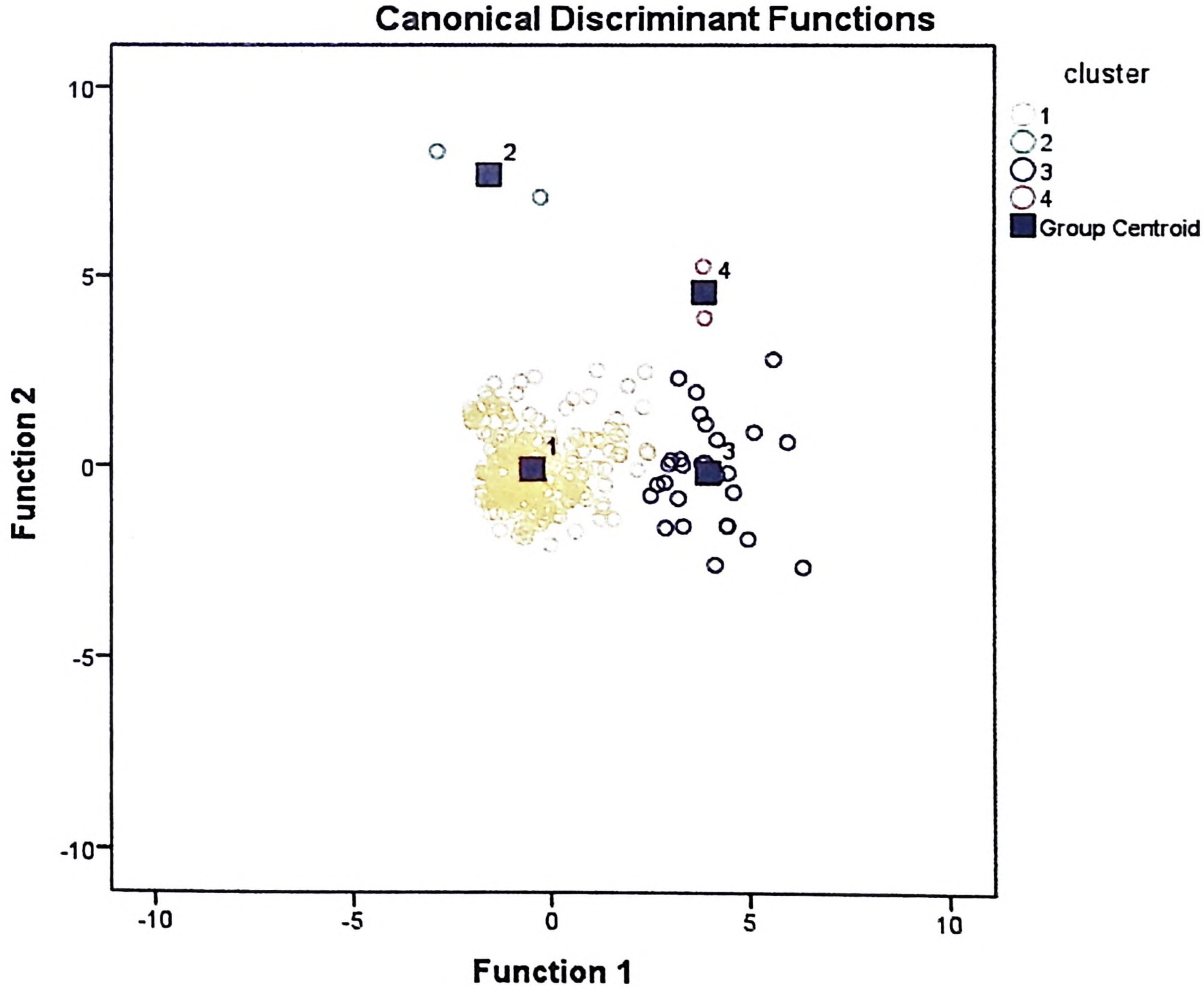

Figure 5. Plot for Discriminant Function Analysis (DFA) using PCA values for 270 vocalisation data from the Indian wolf. Different colours represent different call type.

The whisker box plot represents the variation among acoustic variables within the four identified call types (**Figure 6**). Call type 1 had the longest duration (5.214±2.49 Sec) whereas call type 2 showed the shortest duration among the four recognised groups (0.4±0) (N=2). Type 2 calls also have high-frequency modulation (37.296±4.601) (variation in frequency per unit time). However, frequency variation (around the mean) is highest in type 3 calls (18.778±3.587) (**Table 4**).

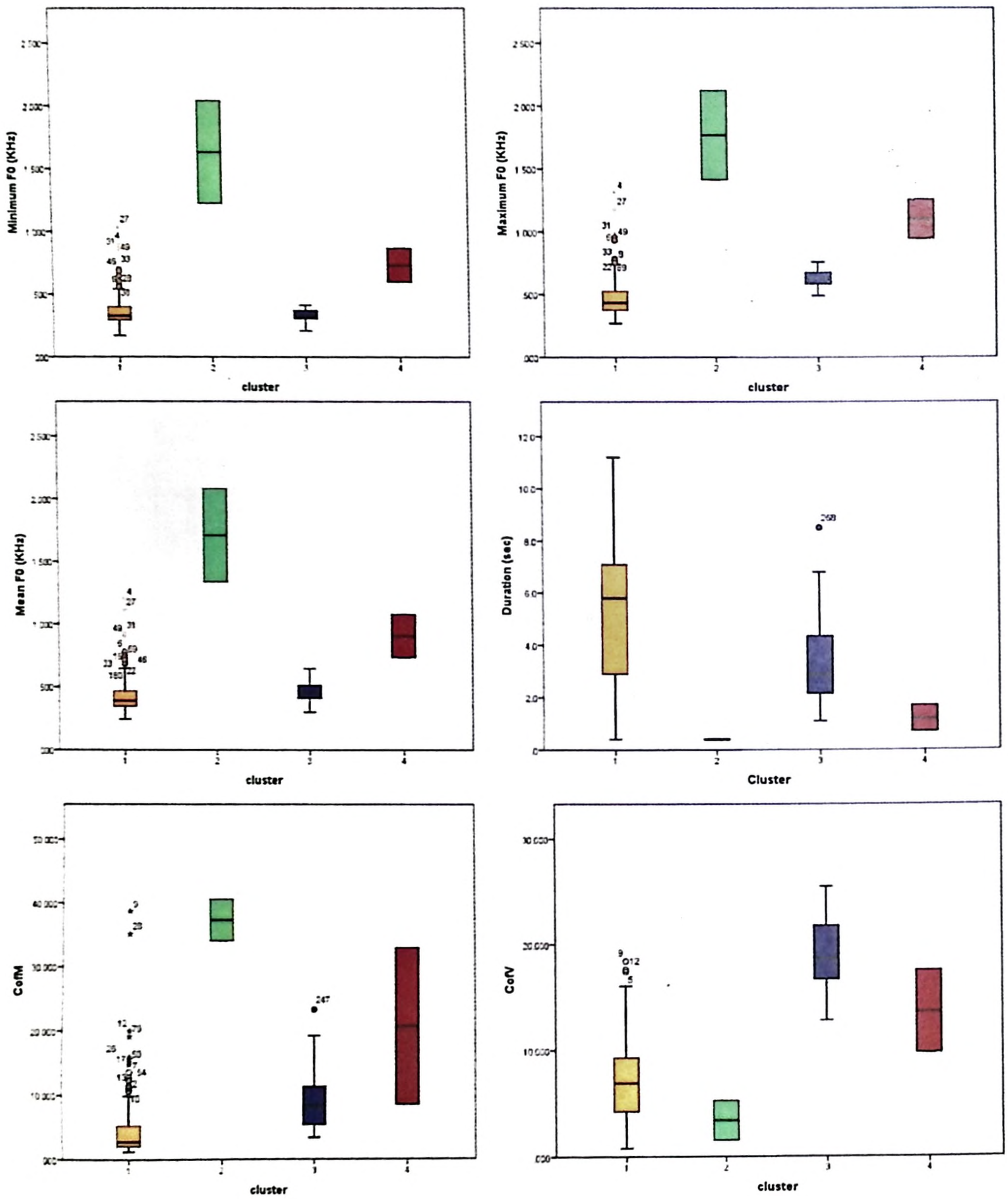

Figure 6. Box plot of variation among acoustic variables between different call type. *a. Variation among minimum frequency. b. Variation among maximum frequency. c. Variation among Mean frequency. d. Variation among duration of the call. e. Variation among coefficient of frequency modulation. f. variation among coefficient of frequency variation.*

Table 4. Variation among important acoustic variables within the four identified call types.

Cluster	Min f_0 (Hz)	Max f_0 (Hz)	Mean f_0 (Hz)	Range f_0 (Hz)	Duration (Sec)	Co-fv	Co-fm
1	359±116	469±141	422±126	110±65	5.21±2.49	7.17±3.689	4.444±4.463
2	1632±578	177±5	1708±524	137±77	0.4±0	3.407±2.632	37.296±4.601
3	327±51	623±77	461±83	295±50	3.47±1.85	18.778±3.587	9.071±4.802
4	733±190	1100±220	906±242	367±29	1.2±0.70	13.649±5.526	20.694±17.347

2.4 Discussion

This study provides a quantitative assessment of the vocalisations of the Indian wolf subspecies. Our results show that there are four statistically classified groups of Indian wolf vocalisations based on ten captive individuals and nine free-ranging Indian wolf packs. Though the Four to Six solution groups showed a narrow difference in their average silhouette values based on *silhouette plot* analysis, the four cluster solution was found to be the most significant based on the global maxima. This characterisation of vocalisations provides a first step to evaluating the function and contextual use of different types of vocalisations in these canids.

The first, most prolonged (5.214±2.49 sec) call type in our dataset is identified as a howl (**Figure** 7a). The fundamental frequency of the howl ranged from 359 Hz (±116) minimum to 469 Hz (±141) maximum (N=238). Despite having a smaller body size, the mean fundamental frequency of the Indian wolf howl (422±126 Hz) was similar to the mean fundamental frequency of other wolf subspecies reported in previous studies (2017). This contrasts previous research that described Indian wolves as having a higher mean frequency in howls (Hennelly et al., 2017). Our study's large sample size of individuals and use of a classification model to statistically discriminate vocalizations may have aided in excluding other vocal types – such as howl barks – in our analyses to robustly describe Indian wolf howls. Additionally, variation in howl acoustic structure has been suggested to be partly individual-specific, which may be due to a combination of differences in body sizes, age class, or gross anatomy (Palacios et al., 2007; Root-Gutteridge et al., 2014b, 2014a; Theberge & Falls, 1967; Tooze et al., 1990; Watson et al., 2018). For example, the mean fundamental frequency of 11 Iberian wolf individuals was reported to range from 332Hz (±47) to 666Hz (±60) (Palacios et al., 2007). This high acoustic variation associated with individual wolves highlights the importance of having a large enough sample size of individual wolves to robustly describe vocal types and assess individual-specific variation within a population. To further understand the influence of body size on wolf howl acoustic structure, it would be important to identify howls using a classification-guided approach across all vocalization data of various subspecies as well as incorporating information of each howl's associated wolf weight and individual's identity.

Since the howl is the most detectable vocalisation used in long-range social cohesion and territorial advertisement (Kershenbaum et al., 2016; Mech, 1981), our high howl sample size should not be considered as the most frequent vocalisation. Barking-howl, which was mentioned by many authors as a common type of mix vocalisation in wolves (Joslin, 1966; Mech, 1981; Passilongo et al., 2017), falls under the same cluster along with howling (**Figure 7b**). From our field observations, wolves bark in defence to an immediate threat. In one such occasion, the she-wolf of a pack started barking at nearby villagers to protect her pups and did not stop until all the three pups ran away to a safer distance from the villagers.

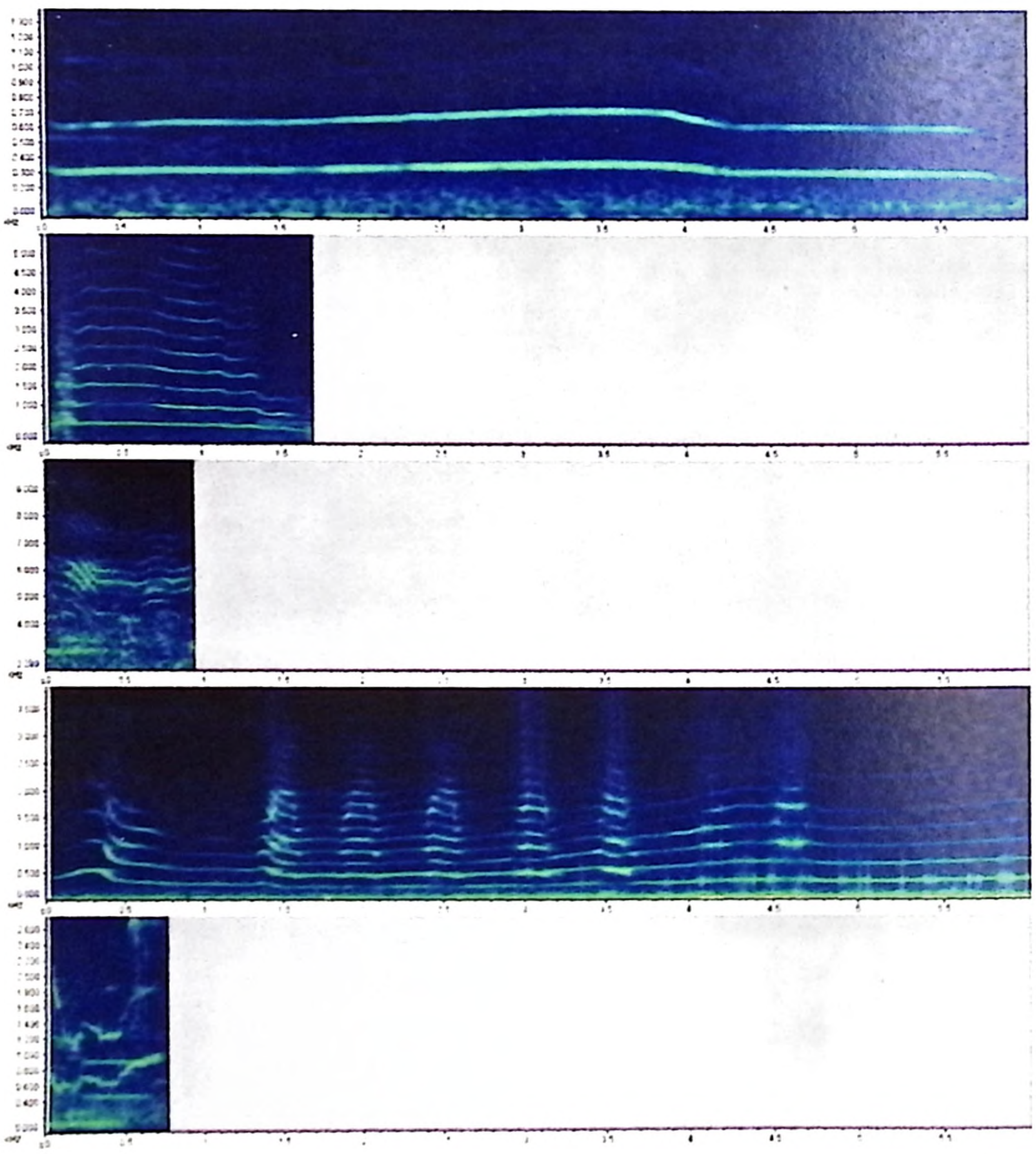

Figure 7. Spectrograms of different types of vocalisations of the Indian wolf. *a. Howl (type 1); b. Bark-Howl (type 1 subtype); c. Whimper (type 2). d. Social Squeak (type 3); e. Whine (Type 4);*

While the howl has been extensively studied in its behavioural function and variation across subspecies (Hennelly et al., 2017; Kershenbaum et al., 2016), less is known about short-range communication among wolves. Our study has identified and described three short-range communication call-types found in Indian wolves. Corresponding to our results, the second call type has the highest frequency modulation (37.296±4.601) and is commonly known as a whimper (**Figure** 7c). The whimper is low intensity but high-pitched sound that is used for short-distance communication among pack members (McCarley, 1978; Mech, 1981) (mean fundamental frequency = 1708±524 Hz). This short duration (0.4±0 sec) vocalisation is reported to be associated with submissive or friendly greeting behaviour (Joslin, 1966; Mech, 1981). Since it is not audible from more than one to two hundred yards away (Joslin, 1966), our dataset contains only a few observations (from different packs) of this type of call (N=2). While our study provides some initial insight into the acoustic structure of this vocalisation, further sampling will be needed to characterize the acoustic structure of the whimper robustly. The third group of Indian wolf vocalisations can be termed as *'social squeak'* (**Figure 7c**), following observations by previous studies (Mech 1981, Crisler 1959, Fentress 1967). This high-frequency variable vocalisation (18.778±3.587) in the Indian wolf is similar to 'talking', which was defined as 'hovering around one pitch' (1959, p. 149). The social squeak is considered to be context-dependent, with variation within the call type being dependent on differing social interactions among individuals (Weir, 1999). Otherwise, there is little known about its function in wolf packs and if it's a common communication across different canid species and within domestic dogs. Our results suggest that the social squeak has a minimum frequency of 327 Hz (±51) to Maximum of 623 Hz (±77) for Indian wolves (N=28).
Lastly, our fourth vocal group we identified as the whine (**Figure** 7e), which is characterized as a short duration vocalisation (1.2±0.707 Sec). The whine is mainly used during stressful situations, such as pack separation and/or intra-pack conflict (Faragó et al., 2014). Additionally, female wolves have also been reported to whine during the nursing of pups in the den (Goldman et al., 1995). The whine in the Indian wolf (*Canis lupus pallipes*) is longer than the whine reported in Italian wolf (0.13±0.10 sec), which the Indian wolf has comparatively larger body size than Indian wolf (Passilongo et al., 2017). Although our data set is too small (N=2; from two different individuals) to interpret robustly, the mean fundamental frequency of Indian wolf whine (906±242 Hz) has a similar frequency as the Italian wolf (979±109 Hz) (Passilongo et al., 2017).

Arising from the challenges of monitoring elusive and low-density species, acoustic methods for detection and estimating population parameters has become increasingly utilized in wildlife management (Buxton et al., 2018; Stevenson et al., 2015). Early wolf biologists had recognized its effectiveness for detection (Fuller & Sampson, 1988), and further statistical work on howl acoustic structure has improved its ability to monitor wolf populations (Palacios et al., 2016; Papin et al., 2019; Passilongo et al., 2015). Statistically validating wolf howls from other vocalisations using an unsupervised classification technique avoids having a human biased sample of vocalisations for performing subsequent behavioural and statistical analyses, such as for identifying individuals. It is important to note that howls can be context-dependent, in which individuals' howl acoustic structure can vary according to certain behavioural contexts (Watson et al., 2018). Since the howls were recorded from both elicited and spontaneous responses, our study's characterization of the howl should be taken with caution, as it may comprise of multiple context-specific howls.

Further research on a larger dataset of Indian wolf vocalisations can develop a more robust classification of the vocal repertoire of this subspecies. Additionally, we defined call types in our study based on similarity to previously defined call types, such as whimper, whine, and social squeaks (Harrington & Mech, 1978b; Joslin, 1966). Incorporating information on the behaviour associated with these call types would aid in describing and validating the call types in our study. Therefore, statistical classification coupled with behavioural monitoring through a visual recorder is one future avenue of research, which will aid in decoding wolf behaviour in the context of its vocalisation. More broadly, the species within the *Canis* clade vary in their body sizes, social structure, and habitats (Macdonald, D., & Sillero-Zubiri, 2004). The diversity of social complexity and vocal communication across species within *Canis* represents a unique system to address questions on the relationship between vocal communication and social complexity (Holekamp et al., 1999; Manser et al., 2014; Pollard & Blumstein, 2012). Therefore, describing the vocal repertoires of various canid taxa provides a first step into understanding the ecological, social, and phylogenetic factors influencing the diversity of vocal communication within the genus *Canis.*

CHAPTER 3 Identifying unknown Indian wolves by their distinctive howls: its potential as a non-invasive survey method

This chapter has been published

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3.1 Introduction

Accurate population estimates are a critical part of wildlife biology, conservation and inform management strategies (Buckland et al., 1993). Informed management decisions rely on accurate estimates which can be hard to achieve but are critical as the conservation status of any species is dependent on its population size, which is inversely correlated with extinction risk (Mace et al., 2008). Therefore, it is of the foremost importance to have a statistically robust population estimation technique. However, widely used population estimation methods such as camera trapping and sighting-based distance sampling fall short in analysing population trends of certain elusive species or species living in extensive home ranges (Crunchant et al., 2020; Garland et al., 2020). Many of these species are vocally active, which inspired scientists to study the effectiveness of an acoustics-based population abundance model for these species (Kidney et al., 2016; Rhinehart et al., 2020; Thompson et al., 2010; Wood et al., 2020). Acoustic monitoring has long been used to monitor the presence of aquatic animals, amphibians, insects, and birds (Acevedo & Villanueva-Rivera, 2006; Parra, 1992; Petrusková et al., 2016; Riede, 1998; Sanders & Mennill, 2014). The critical advantages of acoustic monitoring are that it can be operative at day and night (Wrege et al., 2017) and detect visually cryptic species or those spread over large home ranges (Kidney et al., 2016; Kimura et al., 2009; Pérez-granados et al., 2019). Like camera traps, passive acoustics devices can operate throughout the day for weeks without intervention, and this perpetual data can be analysed easily with the advancement of methodologies for automating the process (Gibb et al., 2019). Recordings from these devices can be used in calculating a wide range of metrics including acoustic indices(Depraetere et al., 2012; Papin et al., 2019), animal diversity (Depraetere et al., 2012; Wheeldon et al., 2019), animal localisation (Gable et al., 2018; O'Gara et al., 2020; Wilson & Bayne, 2018), and density (Dawson & Efford, 2009; Stevenson et al., 2015) estimation. This density estimation is mostly obtained through Spatially Explicit Capture-Recapture (SECR) that relies on multiple recording stations for Capture-Mark-Recapture (CMR), and instead of 'recapture' with time, it considers 'redetection' in different points in space (Dawson & Efford, 2009; Royle et al., 2013; Stevenson et al., 2015). This methodology is applied to individuals that are not identifiable from their calls (Marques et al., 2013; Stevenson et al., 2015). The conventional CMR model requires identification at the individual level (Adi et al., 2010; Marques et al., 2013), but it provides a robust population estimation(Adi et al., 2010) and much additional information such as home-range, survival rate, animal movement pattern, and population viability analysis (Lettink & Armstrong, 2003). However, the difficulty of successfully identifying unknown individuals from their calls has prevented its use for more species, though new techniques are being developed for some species, including the use of unsupervised classifiers to cluster calls (Clink & Klinck, 2020). Here, we explore the potential of identifying individuals through supervised classification from their vocal features to potentially improve identification to the point where CMR surveys are possible for an elusive and wide-ranging species.

Like other grey wolf subspecies, Indian grey wolves are known for their long-ranging communication via howls (Theberge & Falls, 1967). Howling is a social communication process, vital for the overall behaviour of many canid species (Kershenbaum et al., 2016). It has evolved in wolves to communicate with other group members over a long distance as well as to demarcate their vast territories (Harrington & Mech, 1978a). Due to its high amplitude and low frequency, a howl can travel for six kilometres or more (Harrington & Mech, 1978b; Joslin, 1966; Suter et al., 2016). Hence, an acoustics survey using howling may be more advantageous than a visual survey or other methods, such as snow-tracking (Blanco & Cortés, 2012; Gable et al., 2018; O'Gara et al., 2020; Suter et al., 2016). As vocalisations of wolves were found to be highly variable within and among individuals (Theberge & Falls, 1967; Tooze et al., 1990), the howl is a useful tool to identify individuals (Hull et al., 2020; Root-Gutteridge et al., 2014b; Wasser et al., 2009); thus, wolves are ideal candidates for acoustic monitoring techniques.

Previously the '*Howlbox*', a self-contained active acoustics-monitoring device that broadcasts howls and records the responses automatically, was tested for its capability to detect wolves (Ausband et al., 2011; Brennan et al., 2013). This device was unsuccessful in surveying wolves due to low detection rate as, instead of howling back, the wolves visited the device site without howling, and various technical failures (Brennan et al., 2013). A few studies using passive acoustic devices show the potentiality of successful localisation and monitoring of the grey wolf (O'Gara et al., 2020; Papin et al., 2018). However, these only allowed for presence to be detected and stopped short of individual identification. In contrast, the identification of wolves from their distinctive howls will open an opportunity for more conventional CMR methods (Root-Gutteridge et al., 2014a), and this will improve population estimation without bias and help to measure other ecological variables, such as site occupancy and home-range. With the ability to identify individual wolves from howl recordings, information on population sizes, dispersal patterns, pack composition and the presence of pups could be obtained. These would be used to develop conservation management strategies and to examine population trends with howl surveys conducted over multiple years. Therefore, our study aimed to record howls from Indian wolves (*Canis lupus pallipes*) and test the feasibility of identifying unknown individuals from their howls alone using a supervised classification method.

3.2 Methods

3.2.1 Study Species

Indian wolf, subspecies of the grey wolf is among the keystone species found in the Central Indian landscape (Singh & Kumara, 2006) and reside in arid grasslands, floodplains, and the buffer of dense forests (Dey et al., 2010; Habib, 2007; Jhala & Giles, 1991; Singh & Kumara, 2006). The Indian wolf plays a significant ecological role in controlling ungulate populations in the human-dominated landscapes (Jethva & Jhala, 2004b, 2004a; Morin et al., 2016). The population status of Indian wolves is entirely unknown (Jhala, 2020). It is known that Indian wolves face a major threat from humans as their habitat is increasingly used by humans, and human-wildlife conflict is increasing (Habib & Kumar, 2007). Therefore, time is a critical factor to their conservation. The major challenges for population estimation of the wolf are its vast home range of ~230 km^2 (Habib, 2007) and that they actively avoid camera traps because of camera sound, light, and odour emission (Meek et al., 2014). Since implementing standard population monitoring tools in these landscapes is a tremendous challenge, monitoring their population through howls can be an essential technique. The average fundamental frequency and duration of Indian wolf howls are 422Hz and 5.21sec, respectively (Sadhukhan et al., 2019). Due to its low-frequency range and longer duration, it can be heard from an extended distance like howls of other subspecies (Harrington & Mech, 1978b; O'Gara et al., 2020; Suter et al., 2016).

3.2.2 *Study Site*

The study was conducted on captive individuals of Jaipur Zoo and free-ranging, wild wolves of Maharashtra, India.

Jaipur Zoo is situated at the heart of Jaipur City, Rajasthan, India. Since Jaipur is one of the major tourist destination and capital of Rajasthan, the anthropogenic noise is reasonably high in and around the zoo. All the wolves (n=10) in Jaipur Zoo were offspring of captive-bred individuals except one adult male recently captured from a wild population of Rajasthan.

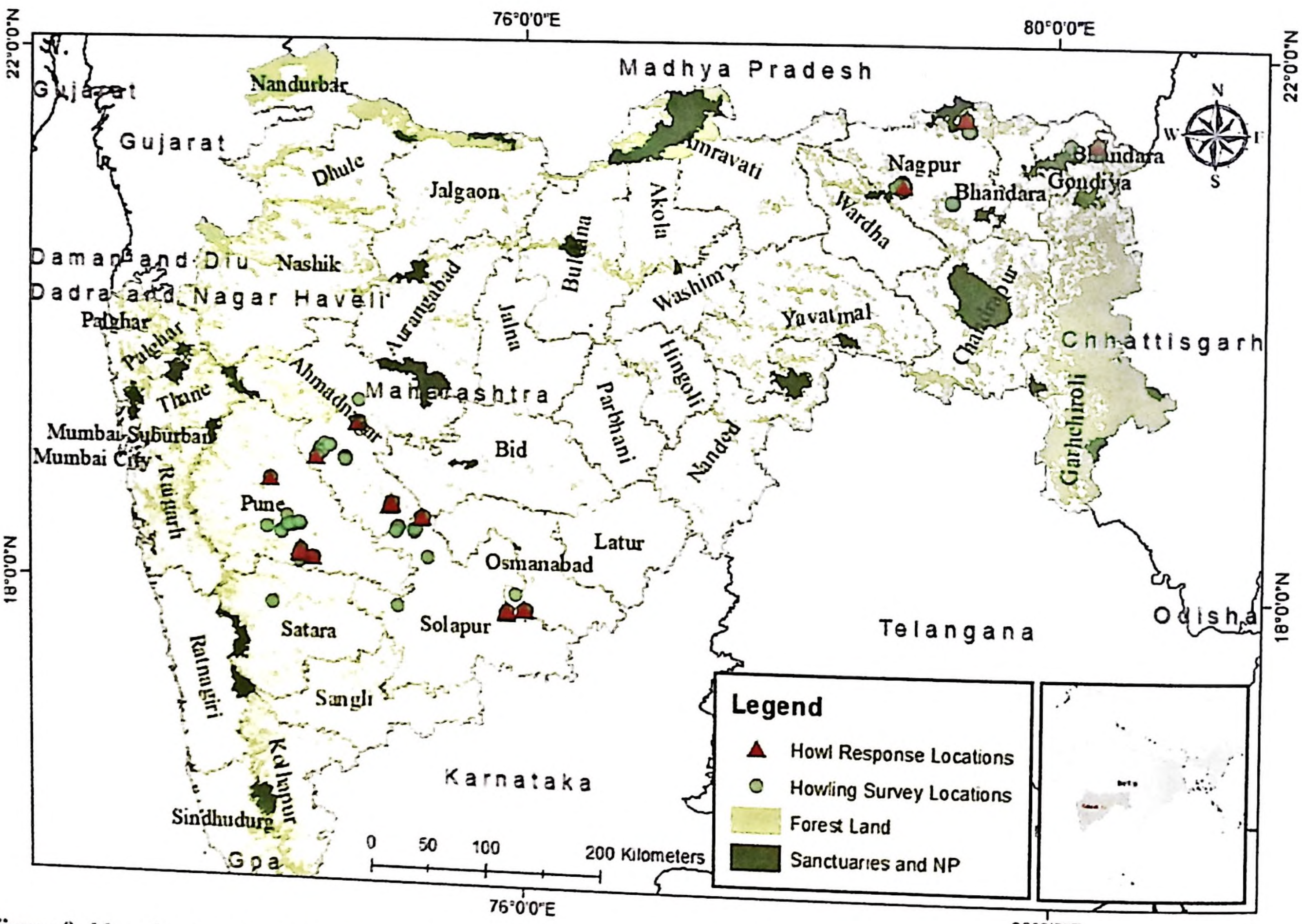

Figure 8. Map showing howling recording locations of the free-ranging wolf in six districts of Maharashtra. Green round bullets indicate the survey locations and Red triangular bullets represent the howling response sites.

The data of free-ranging wild wolves were collected from six districts of Maharashtra. Pune, Ahmednagar, Solapur and Osmanabad (**Figure 8**) districts fall under the semi-arid drought-prone area of the Deccan peninsula Biogeographic Zone (Zone 6) (Rodgers & Panwar, 1988). The dominant habitat type in our sampling areas was *Deccan thorn scrub forests* (Reddy et al., 2015). The terrain is gently undulating with mild slopes and flat-topped hillocks with intermittent shallow valleys, which forms the primary drainage channels. Grassland area is distributed in fragmented patches, creating a mosaic of grazing land, agricultural land and human settlements. Striped hyenas (*Hyaena hyaena*), golden jackals (*Canis aureus indicus*), and Indian leopards (*Panthera pardus fusca*) are the co-predators in this landscape (Habib, 2007; Majgaonkar et al., 2019). Wild prey include blackbucks (*Antilope cervicapra*), chinkaras (*Gazella bennettii*) and wild pigs (*Sus scrofa cristatus*); but a significant part of their diet is domestic livestock (Habib, 2007; Jethva & Jhala, 2004b; Morin et al., 2016).

In Maharashtra, Nagpur and Gondia districts come under the central Deccan Plateau with Tropical dry deciduous broadleaf forests (Reddy et al., 2015; Rodgers & Panwar, 1988). Due to moderate to high rainfall, vegetation is dense in most of the areas. Our sampling areas were mostly packed with open forest and modest density forest. The terrain is generally flat. Nagpur division is surrounded by Many National parks and Sanctuaries. Wolves are primarily found in the buffer areas of these protected areas. Co-predators in those stretches are tigers (*Panthera tigris tigris*), Indian leopards, sloth bears (*Melursus ursinus*), striped hyenas, dholes (*Cuon alpinus*), and golden jackals. Prey species are sambar (*Rusa unicolor*), nilgai (*Boselaphus tragocamelus*), chital (*Axis axis*), chousingha (*Tetracerus quadricornis*), and wild pigs.

3.2.3 Data collection

The howls from the Indian wolves were recorded from November 2015 to July 2016. The howls were recorded during the systematic howling surveys accompanied by the opportunistic and spontaneous recordings of captive and free-ranging wolf howls. Howling surveys were done in the early morning (from 4:30 am onwards) and early evening hours (up to 7:45 pm) [time varies depending on sunrise and sunset]. The survey protocol was adapted from Harrington and Mech (1982). Each howling session consisted of five trials with three-minute intervals. A series of 50-second-long pre-recorded solo howls (from an individual in Jaipur Zoo) was played three times with increasing amplitude; the session was followed by a 50-second-long chorus howl (from three individuals in Jaipur Zoo) in the order of mid and highest amplitude level of the speaker respectively. A 40-watt JBL Xtreme speaker (Harman International Industries, 2014) was used for the playbacks. If howling responses were recorded, the playback session was terminated and repeated after 15 to 20 minutes. All howls were recorded in a single microphone setup, using a Blue Yeti Pro USB Condenser Microphone

(Blue Microphone, 2011) attached with Zoom H4N Handheld Audio Recorder (Zoom Corporation, 2009) with a sampling rate of 44.1kHz and 16-bit depth.

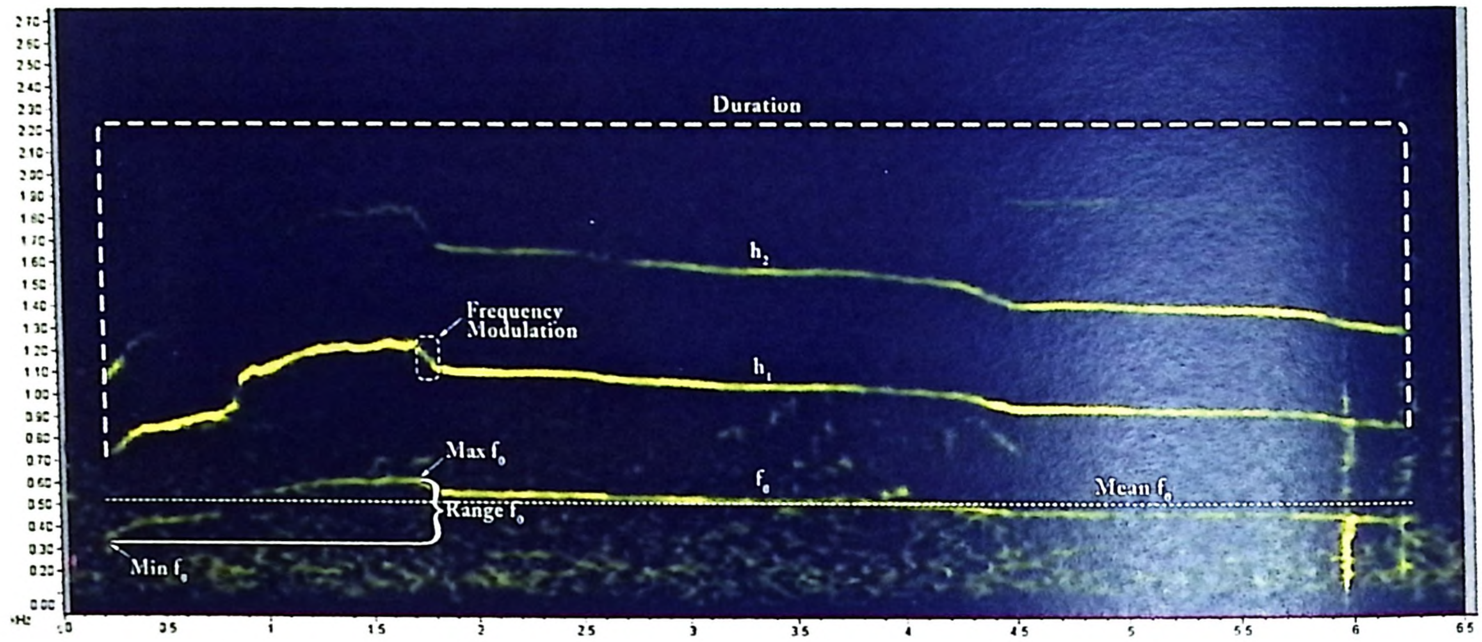

Figure 9. Spectrogram of Gangewadi Wolf howl showing how different variables were measured.

Table 5. Thirteen different variables that were measured from the fundamental frequency (f_0) [Lowest frequency of periodic waveform of each howl]

Variable Name	*Definition of Variable*
Min f	The minimum frequency of the fundamental (f_0)
Max f	The maximum frequency of f_0
Range f	Range of f_0; f_0= Max f – Min f
Mean f	Mean frequency of f_0 at 0.1 s interval over the duration
Duration	Duration of Howl measured at f_0; Duration = t_{end}- t_{start}
$Abrupt_{0.025}$	Number of abrupt changes in f_0 more than 25Hz at single time step (0.1sec)
$Abrupt_{0.05}$	Number of abrupt changes in f_0 more than 50Hz at single time step (0.1sec)
$Abrupt_{0.1}$	Number of abrupt changes in f_0 more than 100Hz at single time step (0.1sec)
Stdv	Standard Deviation of f_0.
Co-fm	Coefficient of frequency modulation of $f_0 = \Sigma\|f(t)–f(t+1)\|/(n–1)$ X 100/Mean f_0
Co-fv	Coefficient of frequency variation of f_0 = (SD/mean) X 100
Pos Min	Position in the howl at which the minimum frequency occurs = (time of Minf)/Dur
Pos Max	Position in the howl at which the maximum frequency occurs = (time of Maxf)/Dur

3.2.4 *Feature extraction*

The howls were sorted, and spectrograms were generated using a *Discrete Fourier Transform* (DFT) algorithm in *Raven Pro 1.5 software* (Bioacoustics Research Program, 2014). *Discrete Fourier Transform* (DFT) algorithm transforms the same length sequence of equally spaced sample points (N, where N is a prime number) with circular convolution being implemented on the points (Rader, 1968). All the spectrograms were produced using *Hann windows* at the rate of 1800 samples on 35.2 Hz 3dB filter (**Figure 9**). Only recordings with low levels of background noise and without any overlapping sounds, where the howls were clearly visible as contours, were selected for further analysis. Spectral images were digitised using *Web Plot Digitizer Software* (Rohatgi, 2017). Thirteen different features (**Table 5**) were measured from the digitised value by using Microsoft Excel. The details methodology is represented in **Figure 10**.

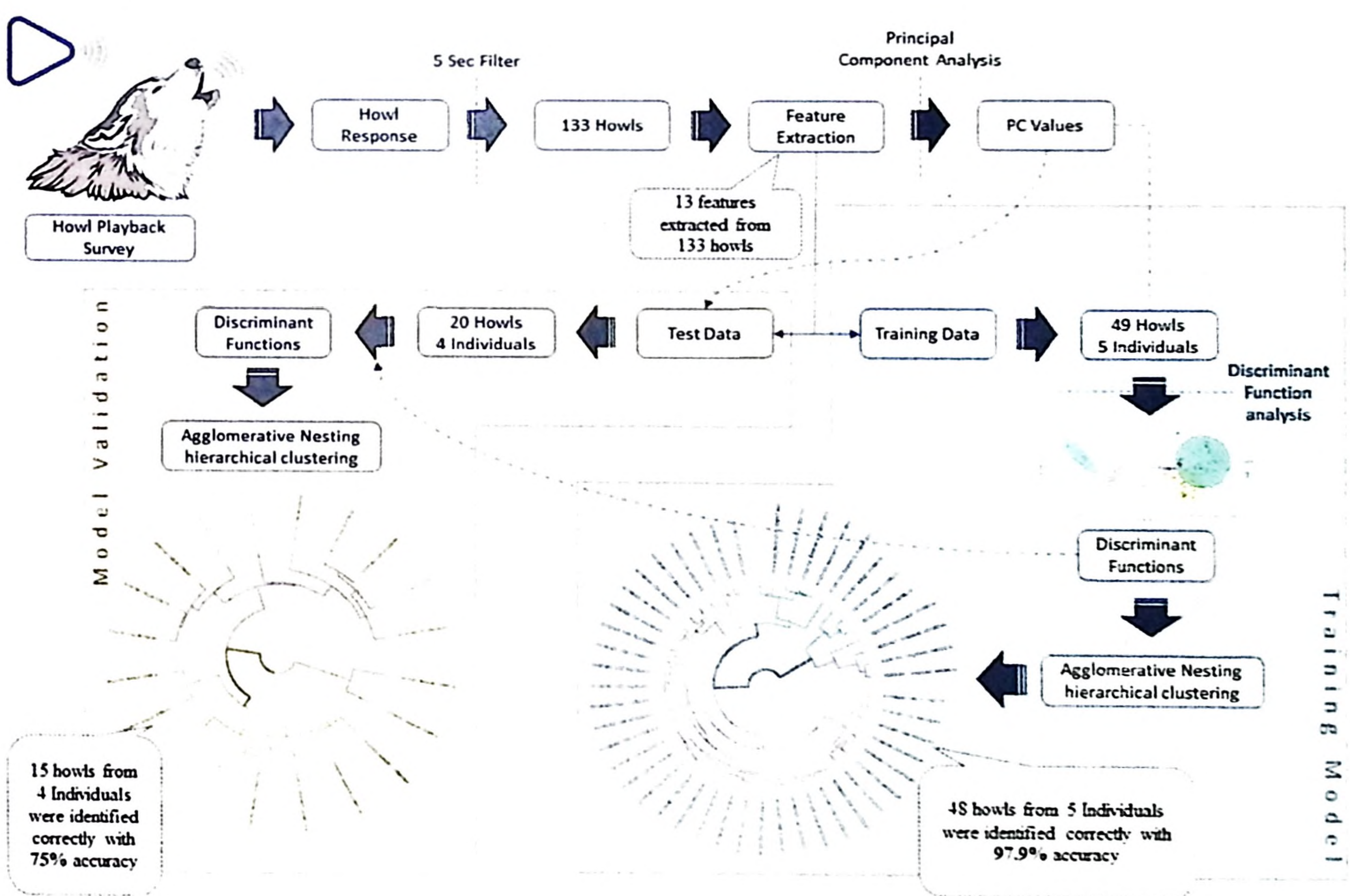

Figure 10. The pictorial representation of methodology for identifying unknown Indian wolves by their howls

One hundred and thirty-three howls that were longer than 5-seconds were used for further analysis, with more than ten individual wolves included. The 5 seconds cut off were chosen to avoid social squeak calls that are very similar to howl but shorter ($\overline{x} = 3.87$ sec) and high-frequency variable calls, described by Sadhukhan et al (2019). Also, the longer howls might contain more identification features than the shorter howls do. *Principal Component Analysis* (PCA) was conducted on measured parameters of 133 howls to reduce the dimension and emphasise the variation between each howl. Out of 133 howls, only 69 howls were identified to an individual. The 69 howls were from nine wolves with known identities: three were captive wolves and six wild, free-ranging wolves, which were identified from their visual features when they were howling in front of the observer and thus howls could be attributed to them individually. The data was further subdivided into training and test datasets. Forty-nine howls from five individuals (2 captives; 3 wild) were used as the training data, and 20 howls from four different individuals (1 captive, 3 wild) as test data to ensure the validity of the method (**Table 6**). Since the known wolf howls were used test data never used in building model, it provides 'unbiased sense of model effectiveness' (Kuhn et al., 2013).

Table 6. Table showing the information on each individual wolf and their capture date with the number of howls were used in this analysis.

Training/Testing	Wolf name	Captive/Wild	Capture Date	No. of Howl
Training Data (n=49)	BMT.SA1	Wild	20/12/2015	5
	CG1.A1	Captive	06/11/2015	3
			08/11/2015	6
	CG2.A1	Captive	05/11/2015	8
			07/11/2015	11
			08/11/2015	9
	GWD.A	Wild	03/02/2016	4
	NNJ.A	Wild	30/01/2016	3
Test Data (n=20)	BMT.A	Wild	19/12/2015	4
	BMT.SA2	Wild	20/12/2015	4
	CG2.A2	Captive	07/11/2015	7
	NU.A	Wild	28/04/2016	5

3.2.5 Discriminant Function analysis

Linear *Discriminant Function Analysis* (DFA) was performed with 49 howls from five individuals (training data) using seven PCA values that contributed more than 5% variation (**Table** 7) [The cut off value was chosen from scree plot]. The objective of DFA was to construct the linear combination of independent *principal component variables* (PC1- PC7) that will discriminate howls of different individuals. The howls were plotted with discriminant functions at two-dimensional space followed by the group prediction (**Figure 11**).

Table 7. Table showing the percentage of variation each Principal Component (PC) accounts for. *Frist seven PC function (marked as bold) contributed 94.8 % in describing the variable.*

	Component Importance (%)
PC1	**41.2**
PC2	**16**
PC3	**10.5**
PC4	**8.1**
PC5	**6.8**
PC6	**6.5**
PC7	**5.7**
PC8	2
PC9	1.7
PC10	1.1
PC11	0.4
PC12	0
PC13	0

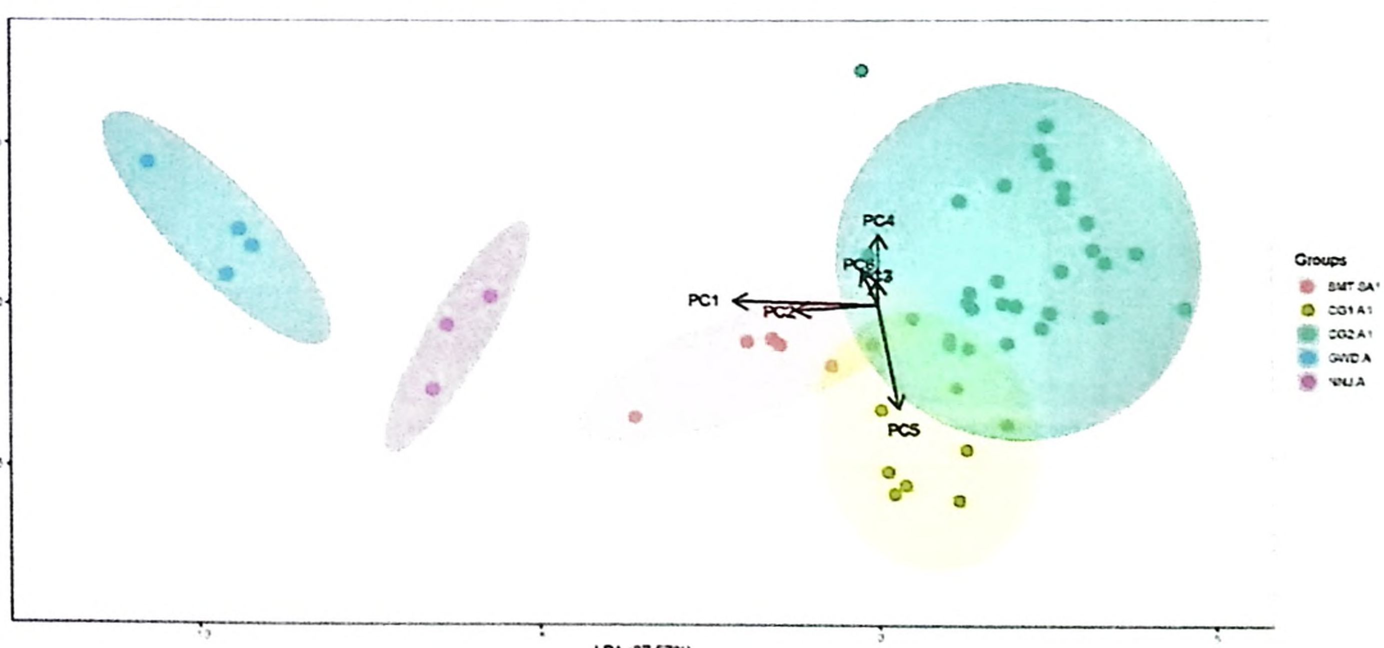

Figure 11. Figure showing a two-dimensional plot of discriminant function analysis using LD1 (Linear Discriminant) and LD2. *Each colour represents each wolf. 100% accuracy was achieved in identifying 49 howls from five Indian wolves.*

3.2.6 *Hierarchical Clustering*

To test the success rate of identifying different individuals from their howls with *Linear Discriminant* (LD) score, an *Agglomerative Nesting hierarchical clustering* (AGNES) was executed on 49 howls (training data) that were used in DFA. AGNES initially considers each howl as a different cluster and use a '*bottom-up*' algorithm to join different clusters based on the similarities (Kaufman & Rousseeuw, 2009). The analysis was performed in R using *'agnes'* function in the package *'dendextend'* and *'manhattan'* metric was used to build the cluster (Galili, 2015). The same analysis was performed on the test data to determine the accuracy of identifying unknown individuals and estimating the number of wolves from their howls. While the test data contained howls from known individuals, the wolves' identities were not included in the model. The variables of these 20 howls were calculated from the equation of DFA of 49 howls for cluster analysis.

3.3 *Results*

3.3.1 *Dimensions reduction to emphasis on variation among howls*

Seven *Principal Components* (PC) that explained more than five percent of the variance each were generated from 13 scalar variables (**Table 5**). These seven PCs together explained 94.8% variance among different howls (**Table 7; Figure 12**). SD of the fundamental frequency (f_0), Frequency (f_0) range, Maximum f_0 and the number of abrupt change (>25 Hz) were the most important contributing factors for building PC1 which contributed 41.2% explaining the variable (**Table 8; Figure 12**).

Table 8. Details of individual identification accuracy using Hierarchical Clustering on testing data (20 howls from four individual). 15 out of 20 howls were identified correctly with the accuracy of 75%.

		Predicted Group Membership				*Identification Accuracy*	*Total*
	Individuals	BMT.A	BMT.SA2	CG2.A2	NU.A	*(correct/total)*	
count	BMT.A	2	1	1	0	2/4	
	BMT.SA2	0	4	0	0	4/4	**15 / 20**
	CG2.A2 .	0	0	7	0	7/7	
	NU.A	0	1	2	2	2/5	
percent	BMT.A	50	25	25	0	50	
	BMT.SA2	0	100	0	0	100	**75 %**
	CG2.A2	0	0	100	0	100	
	NU.A	0	20	40	40	40	

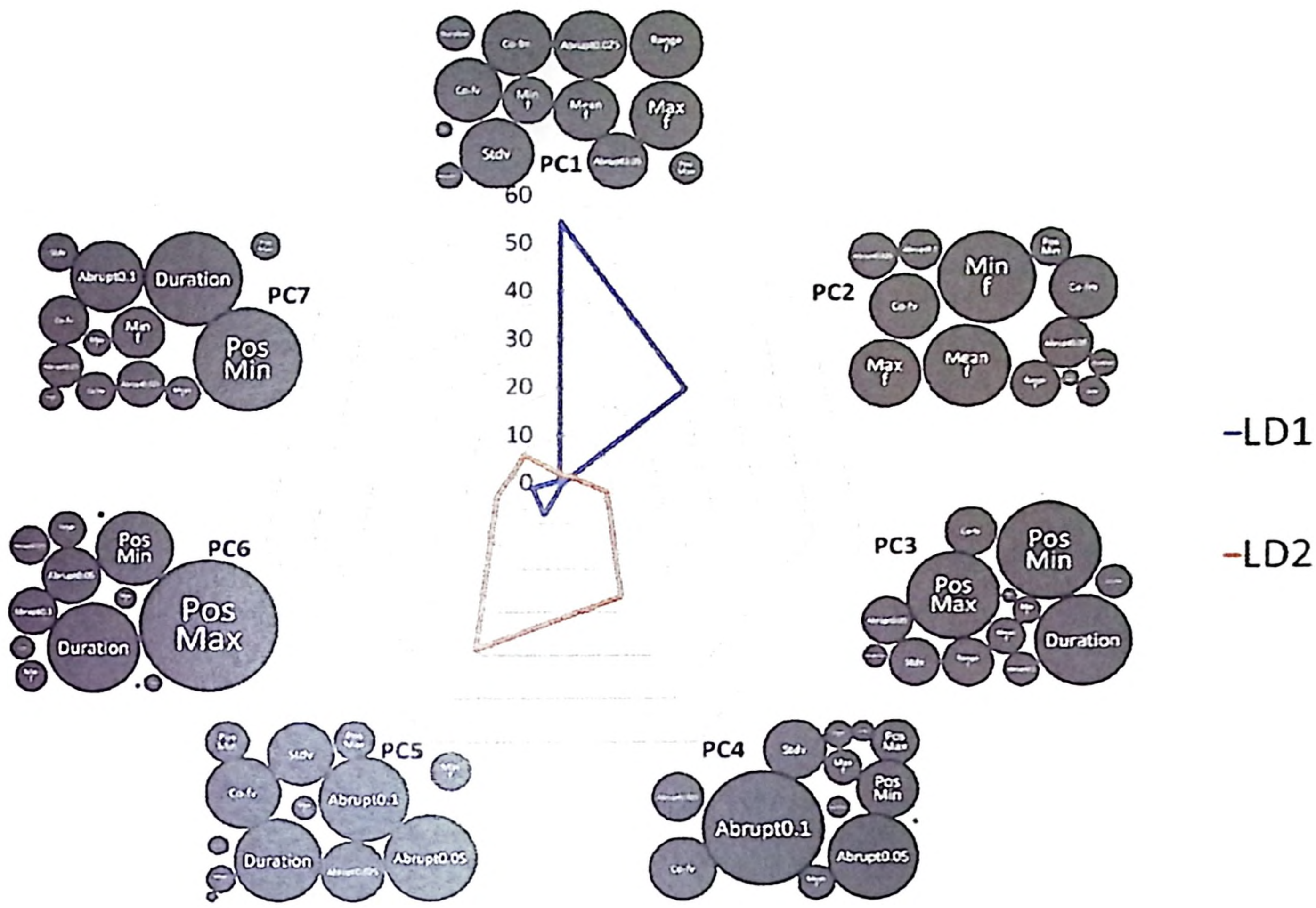

Figure 12. ***The spider web bubble plot is describing how the Simple Scalar Variables (SSV) are ultimately contributing to two LD functions through PC values.*** *The bubble size of each SSV represents the contribution for building each PC function. The blue line represents LD 1, and Orange represents LD2. Since PC1 and PC2 contribute 85% for LD1, the most important SSVs are Stdv f_0, Min f_0, Max f_0 and Mean f_0. Similarly Duration, Abrupt changes, Co-fv contribute the most in building the LD2 function via PC4 and PC5. LD1 was best defined by the different fundamental frequency factors, while LD2 was best defined through the shape of the frequency contour. Therefore, the critical factors for individuality were encoded in X and Y variables.*

3.3.2 *Building Discriminant Function to emphasis on howl variation among different individuals*

The objective of DFA was to build an equation that discriminates the howls of different individuals. The LD score also highlights the variation among howls from different individuals. DFA achieved 100% accuracy in identifying five individuals from 49 howls (**Figure 11**). As the first two *Linear Discriminants* (LD1 and LD2) were responsible for 96.2% of the variance to differentiate between howls of different individuals (LD1 = 87.57% and LD2 = 8.63%), we calculated LD1 and LD2 for rest of the howls using the same function (equation) from last

DFA. PC1 and PC2 contributed 85% in building LD1; PC4 and PC5 are the most crucial factor (65%) for LD2 function (**Figure 12**).

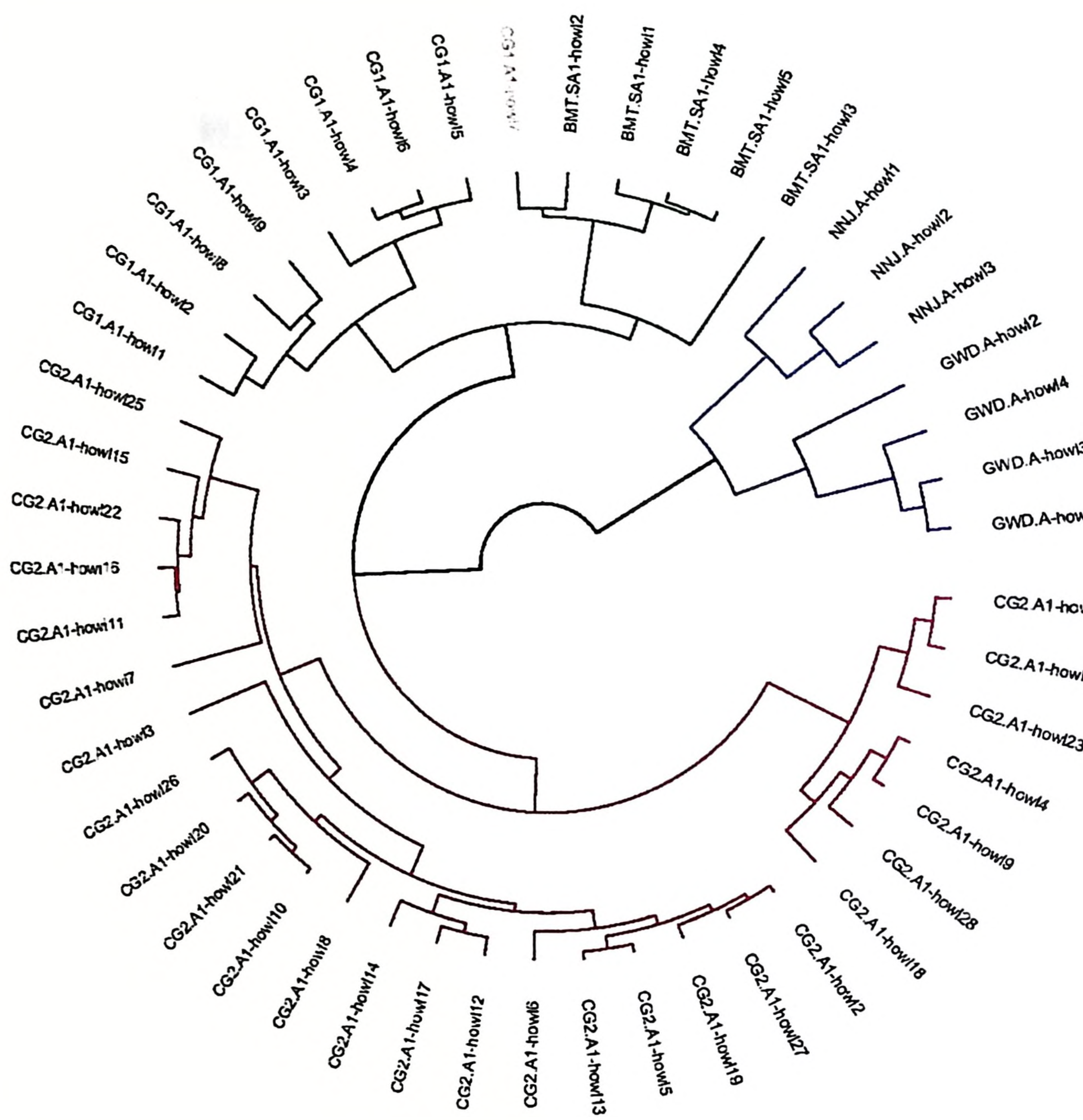

Figure 13. Hierarchical Clustering of 49 howls from five individuals. *These 49 howls were used in training the data. 48 howls were identified correctly with the accuracy of 97.9%. The wrongly identified howl is marked in red.*

3.3.3 Identifying Individuals from their howls in testing dataset

First, we tested AGNES on the training dataset (49 howls from 5 individuals) and found 48 howls (~97.9% accuracy) were identified correctly at 2.2 clustering scale (**Figure 13**). When the same analysis was performed on 20 howls of four different individuals to test the accuracy for the non-training dataset, 15 out of 20 howls from (75% accuracy) four individuals were identified correctly at 2.2 clustering scale (**Figure 14; Table 8**). Two howls from wolf BMT.A were misclassified to wolves BMT.SA2 and CG2.A2; Three howls from wolf NU.A were misclassified to wolves BMT.SA2 (1 howl) and CG2.A2 (2 howls) (**Figure 14; Table 8**).

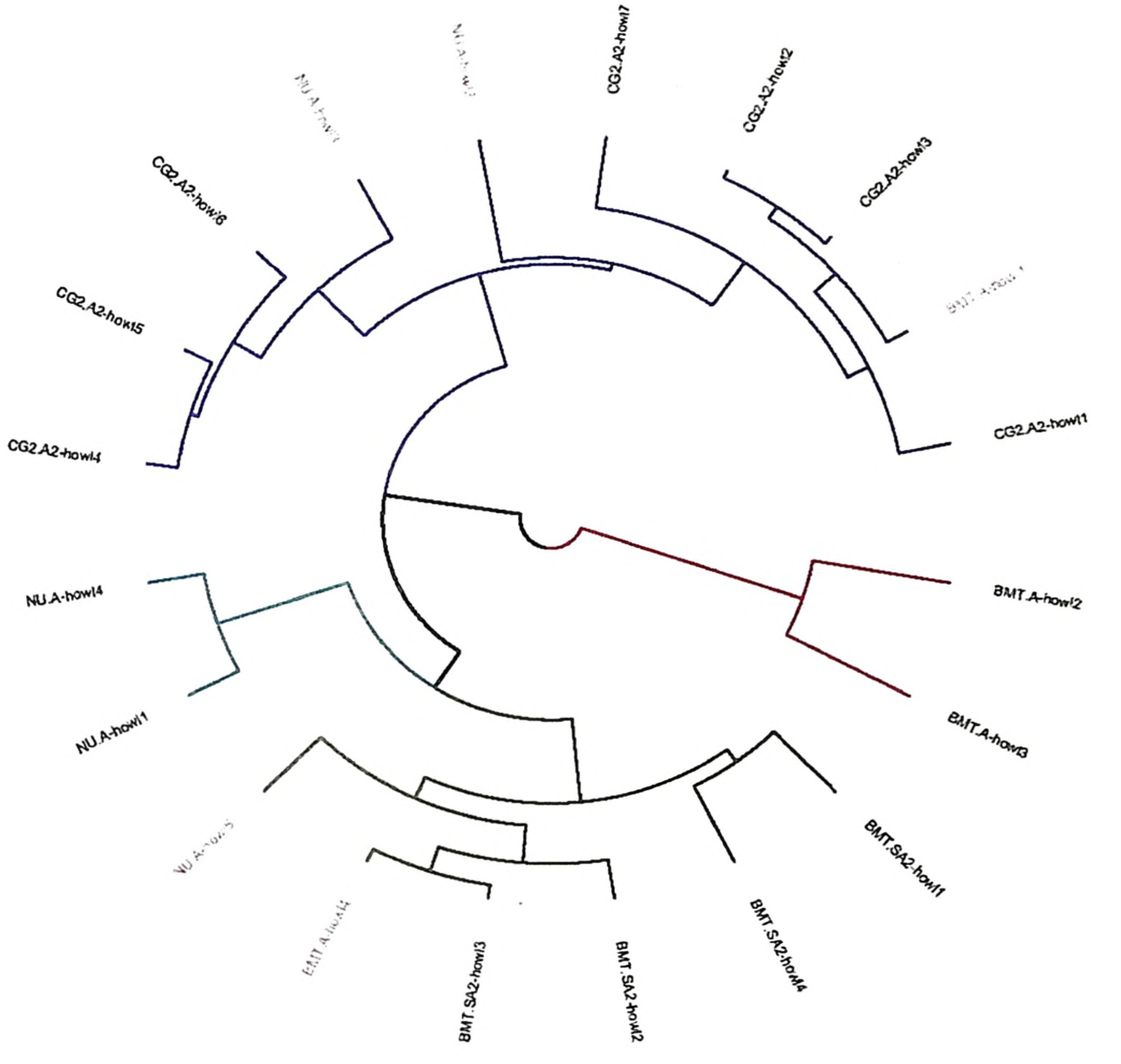

Figure 14. Hierarchical Clustering of 20 howls from four Indian wolves. *None of the 20 howls was used in training the data. 15 howls were identified correctly with the accuracy of 75%, and all the four individuals were identified correctly as different clusters. The correctly identified howls are marked in black, and the five wrongly identified howls are marked in red.*

3.4 Discussion

Here, we presented a new approach to train the classification model, which can identify individuals from their howls and determine the number of wolves present in a certain number of howls, allowing for fine-scale population surveys. In this study, we built an identification model with known training data which was verified with novel test data. The testing data included howls from the known individuals of both captive and wild Indian wolves but independent from the training dataset so that we can cross-check the identification accuracy without bias. The key finding of our study was 97.9% wolf howls were identified correctly from training data, whereas the accuracy of the model on the testing data was 75%. Moreover, we were able to identify four individuals accurately from the testing dataset. The primary significance of this study is that it can be replicated for any other wolf sub-species with a set of a known wolf howls. This study increases the feasibility of wolf pack census using a howling survey (Harrington & Mech, 1982; Suter et al., 2016). Since wolves may actively avoid camera traps (Meek et al., 2014) and photo-identification of wolf requires arduous effort (Galaverni et al., 2012; Garland et al., 2020), identifying wolves from their howls is a big step towards population estimation using CMR.

Although CMR associated with camera trapping provides population estimation without bias for an identifiable animal like a tiger (Jhala et al., 2019), camera trapping has several limitations for non-identifiable and long-ranging species like the wolf (Garland et al., 2020). Other non-invasive methods like DNA-based CMR resulted in biased population estimation due to the animals' non-uniform scent-marking patterns (López-Bao et al., 2018; Morin et al., 2016). However, acoustics based surveys allow vast area sampling with limited resources as compared to camera trapping and other non-invasive methods (Garland et al., 2020). Furthermore, our field observations of wolves have shown that the whole pack typically howls during choruses and that all individuals are acoustically active(Brennan et al., 2013).

For population size estimation through an acoustics-based survey, a combination of CMR and Distance Sampling is required to reduce bias and heterogeneity in detection probability (Laake & Borchers, 2004; Marques et al., 2013). Identifying individual wolves from their howls close this gap of implementing the CMR technique for the population assessment of this elusive and challenging to track species (Kidney et al., 2016; Marques et al., 2013; Stevenson et al., 2015). While a few studies have established that howls carry individuality information (Tooze et al., 1990) and known howls can be distinguished from each other (Palacios et al., 2007; Root-Gutteridge et al., 2014a, 2014b), no study has been successful before in identifying unknown individuals from a set of howls. Furthermore, attempts to count the number of individuals present in a recording have been limited by difficulties in minimising confidence intervals (Papin et al., 2019; Passilongo et al., 2015). There are two ways to identify individual wolves or packs - Supervised clustering and Unsupervised clustering. While supervised clustering requires a set of known training data and cluster validation is straightforward, unsupervised clustering requires ground-truthing before it can be used to monitor populations at a survey level and does not allow individual level CMR or tracking (Clink & Klinck, 2020).

Although DNA-based identification from faecal sampling is more accurate in identifying individuals than our result, it has drawbacks, such as biased population estimation and the increased cost and effort required to collect and analyse the faeces (López-Bao et al., 2018; Morin et al., 2016). Nevertheless, the acoustics-based identification model requires further work to increase its accuracy, though we believe that the successful implementation of this method as a CMR-based supervised population estimation model is already possible.

Wolves mostly live in packs that habitually howl together, and it is challenging to identify the specific wolf that is howling, particularly in choruses. If included and incorrectly attributed to a particular wolf, these howls could lead to erroneous predictions by the model. Therefore, this limited our potential data set to those howls which were conclusively attributed to a known individual, and we dropped many howls, especially the chorus howls, from the analysis to avoid misleading the model. However, larger training datasets from different wolf populations might increase the efficacy of the identification model and verification with more wolf howls conceding better reliability as found for Southwestern Willow Flycatcher (Fernández-Juricic et al., 2009). Thus, our result of 75% may represent a baseline, not a limit, on the accuracy we could achieve. The inclusion of multiple series of howls from every individual would give a more precise result. However, since none of the free-ranging wolves was radio-collared or marked, this was not possible for the wild wolves. Studying howls of collared wolves would help in adding multiple howl sequences from many free-ranging wolves in the training data and may fill this research gap.

This study revealed that the number of wolves present in the recordings could be determined from their howls and the individuality information is sufficient for supervised population estimation through CMR techniques (Clink & Klinck, 2020; Kidney et al., 2016; Marques et al., 2013; Stevenson et al., 2015). Therefore, wolves recorded in one location can be acoustically recaptured at another location, and we can identify them individually. Since our model is exclusively built on fundamental frequency, changes in terrain or vegetation should not affect the accuracy of the model. The information gained from recapturing wolves across different locations would help in deriving territoriality (home-range) information, and this information is crucial for spatially explicit individual-based point process models. This is a clear advancement for developing howling playback surveys as a wolf pack census method. Regular population monitoring will help towards conserving and saving this cryptic species before its population falls beyond a recovery level. Furthermore, since wolf howls can be detected across distances of more than 6km, identifying wolves from their howls also opens up a new opportunity for non-invasive tracking of this species across large landscapes.

3.4.1 Guidelines to implement the methodology on the field: -

We used this methodology to identify individual Indian wolf howl. However, one can use this methodology to identify species, sub-species or individual from their calls. This requires a set of calls to make up the training dataset and a set of calls to make up the testing dataset. We recommend some precautions and step by step guidelines for adapting this method.

I. Before the data collection, one should be cautious about choosing the recorder and data collection methodology. Although we are not definite about the impact of multi-recorder setup in identification accuracy, we recommend using a single microphone set up to keep consistency, especially for individual identification as differences in sensitivity and recording parameters can influence acoustic integrity [See (Root-Gutteridge et al., 2014a, fig. Figure 3)].

II. The multiple groups in the training dataset should be carefully selected to represent distinct group member calls with high confidence (e.g. species/sub-species/individuals), as a single incorrectly identified call in the training dataset can lead the model to erroneous results.

III. The selection of appropriate spectral features is important. While many species encode their identity in the same features, some encoding is species-specific. We tested a wide range of software which fell short in feature extraction for overlapping calls or where background noise was present. The feature description is only as reliable as the extraction. Here, we used *web-plot digitiser* software for spectrogram digitisation. We recommend the use of any semi-automated graph digitiser tool for noisy or overlapping spectral data.

IV. The training data should contain only known groups (multi-species/multiple sub-species/multiple individuals). Each training group should have at least three to five calls and recordings from multiple sessions will increase the accuracy of the model as the animals may have higher intra-individual variation across days than within them. Thus, the higher the intra-individual or intra-group variation, the greater the number of vocalisations and individuals that should be included in the training dataset to make a robust model for the testing dataset.

V. Even though one can choose an unknown dataset as test data, we recommend using a known dataset when originally validating the model. Using multiple test datasets will increase the model's confidence.

VI. We recommend using multiple small batches as test data (50-100 sample of calls) instead of large data to avoid confusion in cluster groups that may represent other variation in the calls.

VII. To allow study replication, we have made our data and codes available in the Supplementary Materials. While the data needs to be replaced for each study, the system of analysis and classification should be robust and replicable.

CHAPTER 4 Silencing the call of the wild – vocal behavioural response of a top predator to Anthropocene in India

4.1 Introduction

Howls are a long-distance vocalisation of the grey wolf (*Canis lupus*) (Joslin, 1966; Sadhukhan et al., 2019). These calls are used to defend their territories (Harrington & Mech 1978b, 1983) and maintain social cohesion and bonding within the pack (Mazzini et al., 2013; Watson et al., 2018). Additionally, the wolf howl significantly impacts associated predator and prey species, which subsequent influence their foraging behaviour (Cooke et al., 2013; Janczarek et al., 2021; Suraci et al., 2016). Therefore, apart from the wolf's role as an 'apex predator', the howl has a substantial ecological role in influencing the lower cascade (Suraci et al., 2016). Changes in their howling behaviour could potentially impact the entire ecosystem. Such behavioural alteration in the Anthropocene has been highlighted recently in a wide range of vocal species, from invertebrates like crickets to amphibians like frogs to large mammals like whales (Halliday et al., 2019; Nedelec et al., 2017; Tennessen et al., 2018; Wale et al., 2013). The impingement of spatial disturbance and noise on vocally active animals may disrupt their parental, territorial, and breeding behaviours (Berger-Tal et al., 2019; Cañadas Santiago et al., 2020; Injaian et al., 2018). Since a large portion of wolf habitats fall within the human-modified landscape (Mech, 2017) and anthropogenic factors may influence their fundamental howling behaviour (Viola et al., 2021).

Wolves are highly adaptable and occupy a wide range of habitats worldwide (Boitani et al., 2018). In most parts of the USA and across the northern hemisphere, wolves were extirpated during the European colonisation and remained in conflict with humans across their habitats (Berger et al., 2001; McNay, 2002; Mech, 2017; Rich et al., 2012). Fragmentation of wolf habitat due to village and agricultural expansion drives wolves to adapt to the human-modified landscape through behavioural alteration (Habib et al., 2021; Mancinelli et al., 2019;

Rio-Maior et al., 2019). Adapting to human-modified land may save the population from extinction but may lead to radical behavioural alteration with unknown outcomes (Ciucci et al., 2020; Ordiz et al., 2013).

The Indian grey wolf (*Canis lupus pallipes*) is the oldest lineage of modern wolves, hence considered an evolutionary significant unit (ESU) (Aggarwal et al., 2007; Hennelly et al., 2021; D. K. Sharma et al., 2004). They depend on smaller to medium size wild prey such as blackbuck (*Antilope cervicapra*), chinkara (*Gazella bennettii*), wild pig (*Sus scrofa cristatus*) and a few others (Habib, 2007; Jethva & Jhala, 2004a; Kumar & Rahmani, 2000, 2008). They primarily inhabit village outskirts and frequently contact humans (Habib & Kumar, 2007; Jhala & Giles, 1991; L. K. Sharma et al., 2019). Adapting to human-modified landscapes, wolves have shifted their food preferences towards domestic livestock acquired via hunting and scavenging (Habib, 2007; Jhala & Giles, 1991; Khan et al., 2022; Kumar & Rahmani, 2000). Indian wolves have modified their ranging pattern (average home range size~210 km^2) and use multiple core areas (2.33±1.52) to cope with the human-altered landscape, and the core areas mostly connect through villages and agricultural patches (Habib et al., 2021; Khan et al., 2022). As a result, wolves face several conservation challenges, such as hybridisation risk with village dogs, den destruction and revenge killing due to livestock depredation (Agarwala et al., 2010; Hindrikson et al., 2012; Kusak et al., 2018; Linnell et al., 2002; Pacheco et al., 2017). Although studies have highlighted various aspects of Anthropocene-linked wolf conservation challenges, alteration in their vocal behaviour in human-altered landscapes remains unexplored.

Long-range howls are the territorial calls of wolves, making them respond to the howl playbacks (Font et al., 2015; Harrington & Mech, 1978a). Consequently, the howling survey is an effective non-invasive tool for studying this cryptic and wide-ranging species (Harrington & Mech, 1982; Suter et al., 2016). A howl contains the identity of an individual wolf (Hull et al., 2020; Root-Gutteridge et al., 2014a; Sadhukhan et al., 2021). Additionally, Studying howling behaviour can reveal various pieces of information such as social behaviour (Biben, 1983; Faragó et al., 2014; Joslin, 1966), ecology (McIntyre et al., 2017), breeding success through the detection of pups (Palacios et al., 2016), and even evolutionary history (Chen & Wiens, 2020; Hennelly et al., 2017; Kershenbaum et al., 2016). To explore how howling behaviour is affected by different factors, I conducted howl surveys on collared and non-collared wolves. I hypothesise that anthropogenic factors such as village distance and human density influence the howl responses. Furthermore, I examine the cumulative impact of factors such as home range, breeding season, time of day and howl type (chorus, solo or duet howls) on the wolf responses. The study highlights the influence of human-modified landscape on the howling responses and how it may adversely impact the whole ecosystem. Additionally, I standardised an efficient howling survey method from our current study's findings, which will significantly aid global wolf conservation.

4.2 *Materials and Methods*

4.2.1 *Study Site*

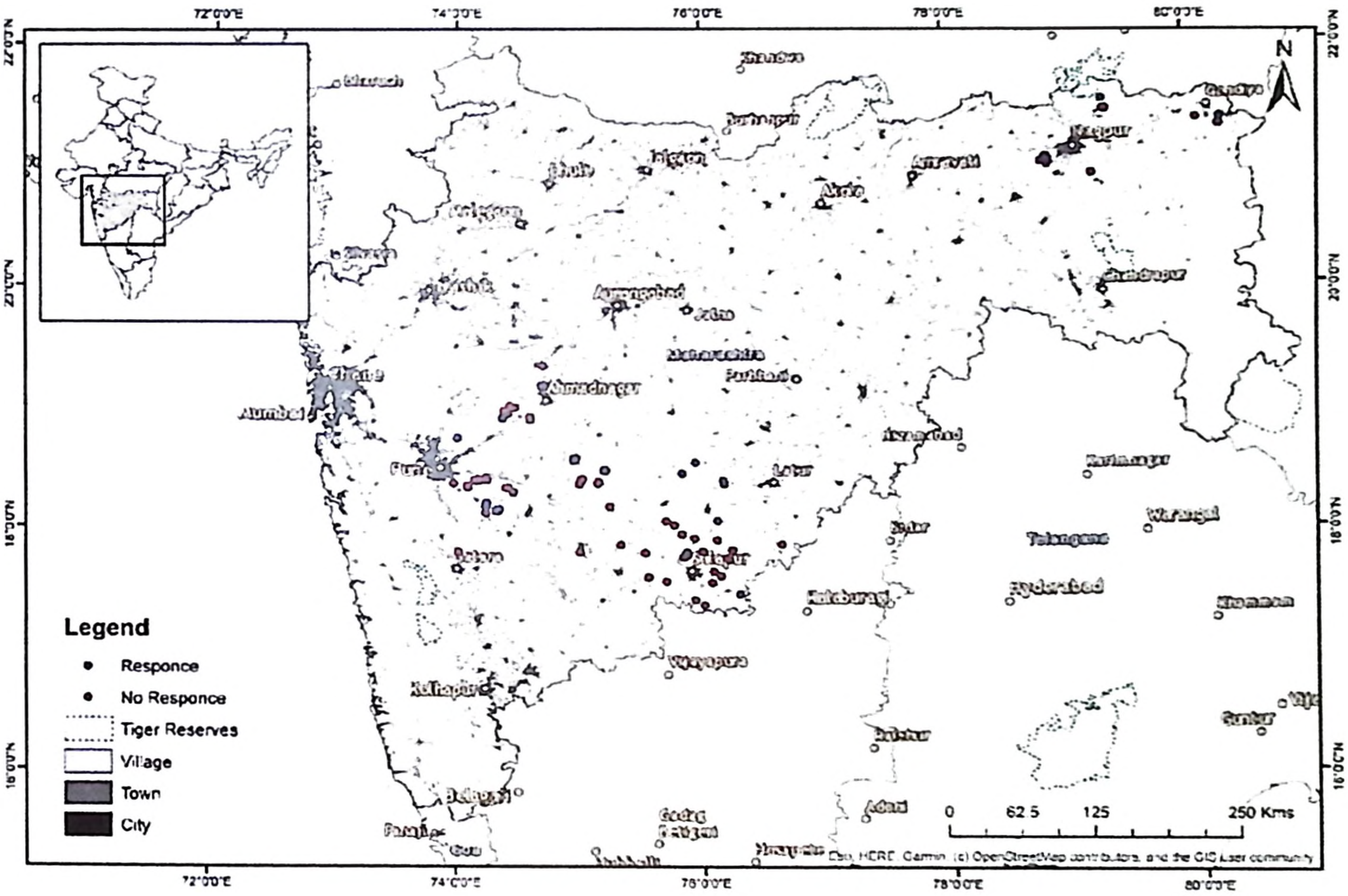

Figure 15. The map represents the howling survey locations of Non-Collared wolves in East and West Maharashtra. *Blue represents the location where I got responses, and pink represents where I did not get any responses. The three shades of black represent cities, towns and villages, respectively, and the Green dotted boundary symbolised tiger reserves. The built-up density in Eastern Maharashtra is lower than in Western Maharashtra, which is visible on the map. Wolves utilise high human-dominated landscapes in West Maharashtra compared to wolves in East Maharashtra, mostly using the forest buffer.*

The study was conducted in the eastern and western parts of Maharashtra, India [**Figure 15**]. Eastern Maharashtra (EM), also known as Vidarbha, is in the central Deccan Plateau with Tropical dry deciduous broadleaf forests with generally flat terrains (Reddy et al., 2015; Rodgers & Panwar, 1988). Vegetation is dense in most of the areas due to moderate and high rainfall. The Vidarbha region is a tiger hotspot and comprises many national parks and sanctuaries (Habib et al., 2018). During our study, wolves were found mainly in the buffer of the national parks and sanctuaries. This landscape has a less built-up area and human density than West Maharashtra (WM).

WM falls under the semi-arid drought-prone areas of the Deccan peninsula Biogeographic Zone (Zone 6) (Rodgers & Panwar, 1988). Deccan thorn scrub forests are the dominant habitat type in the sampling areas (Reddy et al., 2015). The main characteristics of the Deccan peninsula are mild undulating slopes and flat-topped hillocks with intermittent shallow valleys. Wolves were primarily found in grassland and thorn scrub forests highly fragmented by agricultural lands and small villages.

The built-up density for EM and WM was found to be different in the sampling area. The settlement area in EM and WM is $0.22km^2/100\ km^2$ and $0.28km^2/100\ km^2$, respectively (27% higher in WM). The population density (human) of the surveying districts of WM ($1170.1/km^2$) is almost double that of EM ($604.13/km^2$) (*CensusInfo India 2.0*, 2011).

4.2.2 Data Collection

The data was collected in two phases- collared wolf and non-collared wolf. Though the information on collared wolves allows me to include many more vital factors such as animals' home range and distance along with the certainty about their presence, wolf collaring is profoundly high resource-dependent. Therefore, I studied the howling response pattern in collared and non-collared wolve.

In the first phase, the howling survey was conducted on the free-ranging non-collared wolves from December 2015 to December 2019 in the Deccan Peninsula and Vidarbha Landscapes [**Figure 15**]. Surveys were conducted in the potential wolf sites during the early morning (an hour before the sunrise to an hour after the sunrise) and early evening hours (an hour before the sunset to an hour after sunset), considering the peak activity hours (Eggermann et al., 2009; Harrington & Mech, 1982; Šprem et al., 2015). About ten to fifteen free-ranging packs were surveyed. Every howling survey session consisted of five trials and a three-minute interval between the consecutive trials, as standardised by Harrington and Mech (1982). A trial consisted of a 50-second-long pre-recorded playback *solo*, *chorus* or *mixed* (howl sequence altering solo and chorus howl) of both howls. Pre-recorded howls from the Jaipur Zoo were played using a 40W single speaker setup in the order of increasing amplitude in every consecutive trial [See 3.2.3]. The *solo, chorus or mixed howl* is from a captive individual or packs and a single series of chorus howls were played during the entire sampling to maintain uniformity. The time of each playback was documented during the survey, along with GPS locations and other ecological parameters.

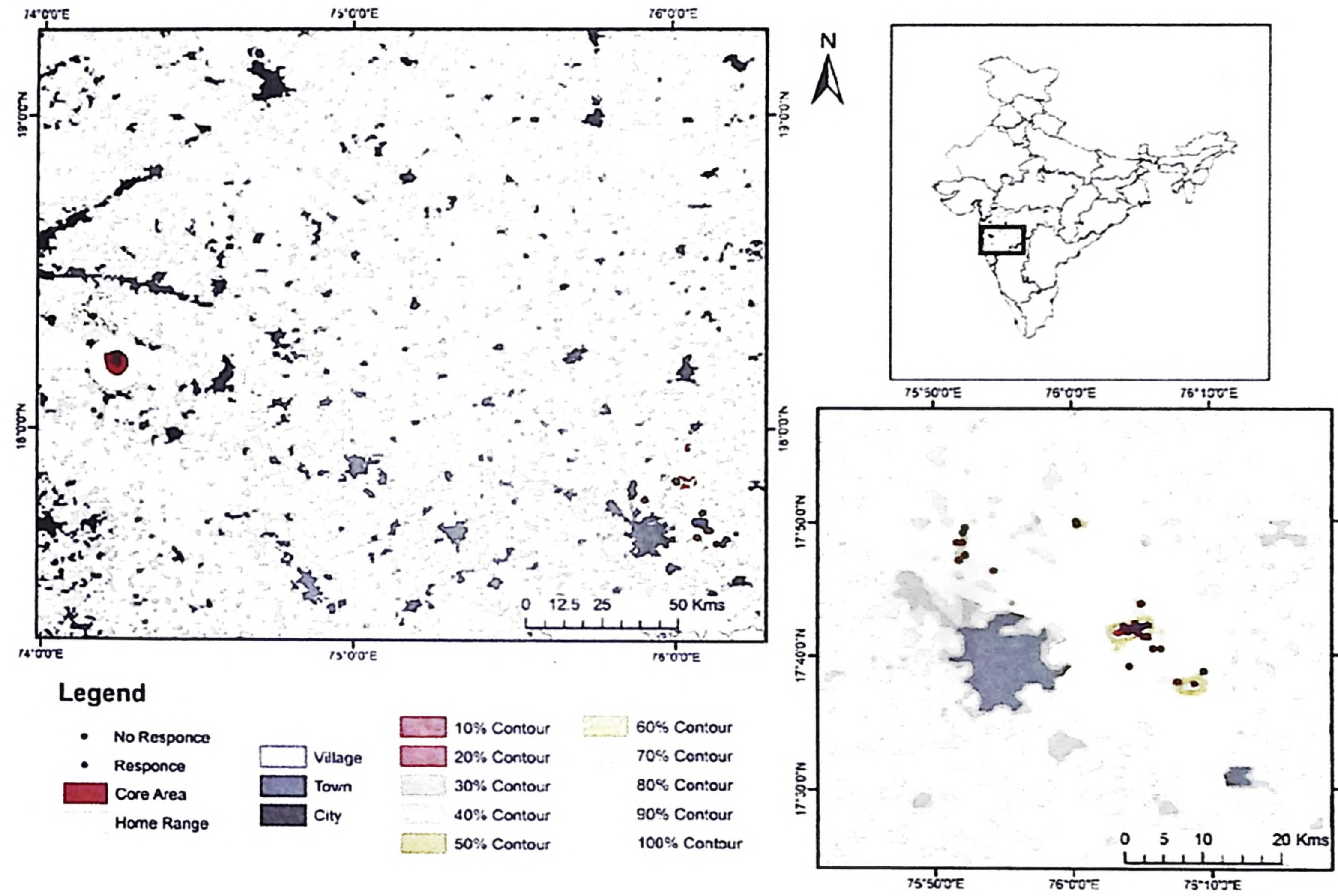

***Figure 16. a)** The map represents a home range of seven collared wolves (Left) [a few individuals own smaller and overlapping home ranges, which may not be visible at smaller image resolution]. Red illustrates core areas (50% utilisation distribution), whereas Yellow explains the home range (95% utilisation distribution). **b)** In the right-side map home range and core area of a collared wolf [Merry (F A)] is represented, where different utilisation zones (0.1-1) are classified into ten classes and described by different colours. Fragmentation in the home range (yellow) is visible, which forces the animal to use multiple core areas. The howling survey locations were represented by blue (response) and pink (no-response) dots.*

In the second phase, the data was collected from January 2018 to July 2019 in the Deccan Peninsula or WM [**Figure 16**]. Seven wolves (five packs) were captured using soft-catch leghold traps and were fitted with satellite radio collars (See Habib et al., 2021). The packs were tracked using a VHF receiver, and howling surveys were conducted once their presence was confirmed within the proximity (<1500 meters). The procedure for playback was the same as in the first phase of data collection, and other information was logged as mentioned in the first phase. Additionally, the animal locations were logged based on Satellite location, and the animal distances were calculated based on the *Pythagoras theorem* (Equation 1).

$$Distance\ between\ observer\ and\ wolf = \sqrt{(X_O - X_w)^2 + (Y_O - Y_w)^2}$$

Equation 1. Formula to calculate the distance between observer and wolf. *X_O, Y_O and X_w, Y_w are the location of the observer and wolf, respectively (in Projected coordinate)*

4.2.3 Data Preparation

The human settlement data of 1 km resolution were acquired from the JRC open data portal (Pesaresi Martino & Freire Sergio, 2016). The human settlement data layer has three classes (rural cells or base, urban clusters or low-density clusters, urban centres or high-density clusters). I converted the human settlement data raster image into a vector file (polygon), and the distances from the edge of the closest rural or urban areas from each howling survey location were calculated in ArcGIS (v10.6) using the nearest feature tool. The human settlement or built-up area density was calculated separately for the sampling area of EM and WM. The Human population density of EM and WM were also evaluated by *Census Info India*, Govt. of India (*CensusInfo India 2.0*, 2011). Howling survey data were categorised into different seasons, October-December (pre-denning), January-March (denning) and April-July (post-denning). The radio-collared individuals were programmed to collect the GPS fix at hour intervals. The home range of seven wolves (three adults and four subadults of five packs) was calculated using the Brownian Bridge Movement Model (BBMM)(Bullard, 1999; Kranstauber et al., 2012). Unlike traditional movement models, BBMM explores movement paths and performs temporal autocorrelation, giving a precise idea about periodic movement patterns and quantifying accurate utilisation distribution (Kranstauber et al., 2012). *Utilisation distance* (UD) is the probability of finding the collared or tagged animal in a specific space over a period of time (Van Winkle, 1975). The home ranges were calculated at different percentile contours (10%-100%) of the UD. Utilisation hotspot spaces of resident animals are known as the *'Core area'* of their home range (Samuel et al., 1985). The howling surveys of collared wolves were conducted in each UD zones (10%-100%) [**Figure 16**] to examine the effect of home range on howling responses. Up to 30%-50% of UD zones are known as *core areas*, and up to 90-95% of UD zones are known as home ranges depending on the study species and methodology (Vander Wal & Rodgers, 2012).

4.2.4 *Data analysis*

4.2.4.1 Non-collared wolf

Generalized Additive Model (GAM) analysis was performed with the package *'gam'* in R (v 4.0.2) to see the cumulative effect of various factors on howling responses (Chambers & Hastie, 1992; Hastie & Tibshirani, 1990). GAM is a generalized linear model where the model relates a univariate response variable (Y=Howl response; binomial variable) with multiple predictor variables (X) (Hastie & Tibshirani, 1990). Seasons (based on the breeding behaviour), distance from the nearest settlement (rural and urban), and sunset or sunrise were the predictor variables used in the analysis.

Since EM and WM comprise different habitat types (vegetation type and human pressure differ), the response pattern might vary between these landscapes. The response rate regarding village distance was plotted in *'ggplot'* in R (v 4.0.2) to understand the response variation between these two landscapes. To test whether EM and WM have different response patterns relating to village distance, I classified data into two groups – i. howling survey conducted within 1200 meters from villages and ii. howl surveys were conducted more than 1200 metres away from villages. The error and 95% confidence intervals were calculated and provided in **Table 9**.

Table 9. The table shows how EM and WM have differential response patterns based on distance from villages.

Distance From Village	Zone	No. of HS	No. of Response	P-value	SE	95% CI
<1200m	EM	66	2	0.030	0.021	0.041
	WM	128	19	0.148	0.031	0.062
>1200 m	EM	31	7	0.226	0.075	0.147
	WM	45	2	0.044	0.031	0.060

4.2.5 *Collared wolf*

To investigate the cumulative effect of different factors (predictor variable) on howling response (response variable, binomial), 'gam' analysis was executed in the R (v 4.0.2) platform. The predictor variables were home range, animal distance to observer, seasonality, distance from the nearest settlement, playback type (solo, chorus or mixed), time of the day, and maximum playback amplitude.

4.3 Results

4.3.1 Factors affecting the howling responses of non-collared wolves

I conducted 264 howl surveys for non-collared wolves in the identified wolf sites in EM and WM. Of the total playback events, 30 howling responses were recorded [**Figure 15**]. The overall response rate (RR) obtained was 9.4%.

Table 10. ANOVA table for Parametric Effects of probable influential factors on howl responses of Indian wolf (non-collared). *Village distance and season are the significant, influential factors of howl responses as they have a cumulative probability value of less than 0.05.*

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Significance Level
Season	2	5.28	2.6401	3.0956	0.04696	$F_{0.05}$
s (village distance)	1	4.085	4.0853	4.7901	0.02953	$F_{0.05}$
Playback type	2	2.64	1.3202	1.548	0.21468	-
Sunset/Sunrise	1	0.538	0.538	0.6309	0.42778	-
Residuals	254	216.627	0.8529			

Table 11. ANOVA table for Nonparametric Effects of the probable influential factor on howl responses of Indian wolf (non-collared). *This table represents the significance level of smooth function, i.e., non-linear relation. Here the smoothening function of village distance represents a cumulative probability value of less than 0.01. That means village distance is non-linearly correlated with howling response.*

Component	Df	Chisq	P(Chi)	Significance Level
s(village distance)	3	12.668	0.005414	$F_{0.001}$

The influence of different predictor variables on howling responses was assessed through *'gam'* and found that breeding season ($F_{2,254} = 3.09$, $p = 0.046$) and village distance ($F_{1,254} = 4.79$, $p = 0.029$) have significant effects on the howl responses of Indian wolf in Maharashtra [**Table 10**]. The howling response was higher in the pre-denning season than in the denning and post-denning seasons [**Figure 17a**]. The effect of village distance over howling response was significantly non-linear ($\chi^2_{3,254} = 12.668$, $p = 0.005$) [**Table 11, Table 10**]. In multifactor 'gam' analysis, wolf responses are consistent up to 1000 meters from villages, but a certain dip was observed after that [**Figure 17c**]. However, the response rate increased after 2000 meters from the village [**Figure 17c**]. The results also show that chorus howls elicit higher response rates than solos or mixed [**Figure 17d**]. However, this is not conclusive due to a significant overlap in the interquartile range of different howl playbacks ($F_{2,254}$ = 1.32, p = 0.21) [Figure 3d]. The result showed no significant difference in response rate during sunrise or sunset ($F_{1,254}$ = 0.63, p = 0.43) [**Figure 17**b].

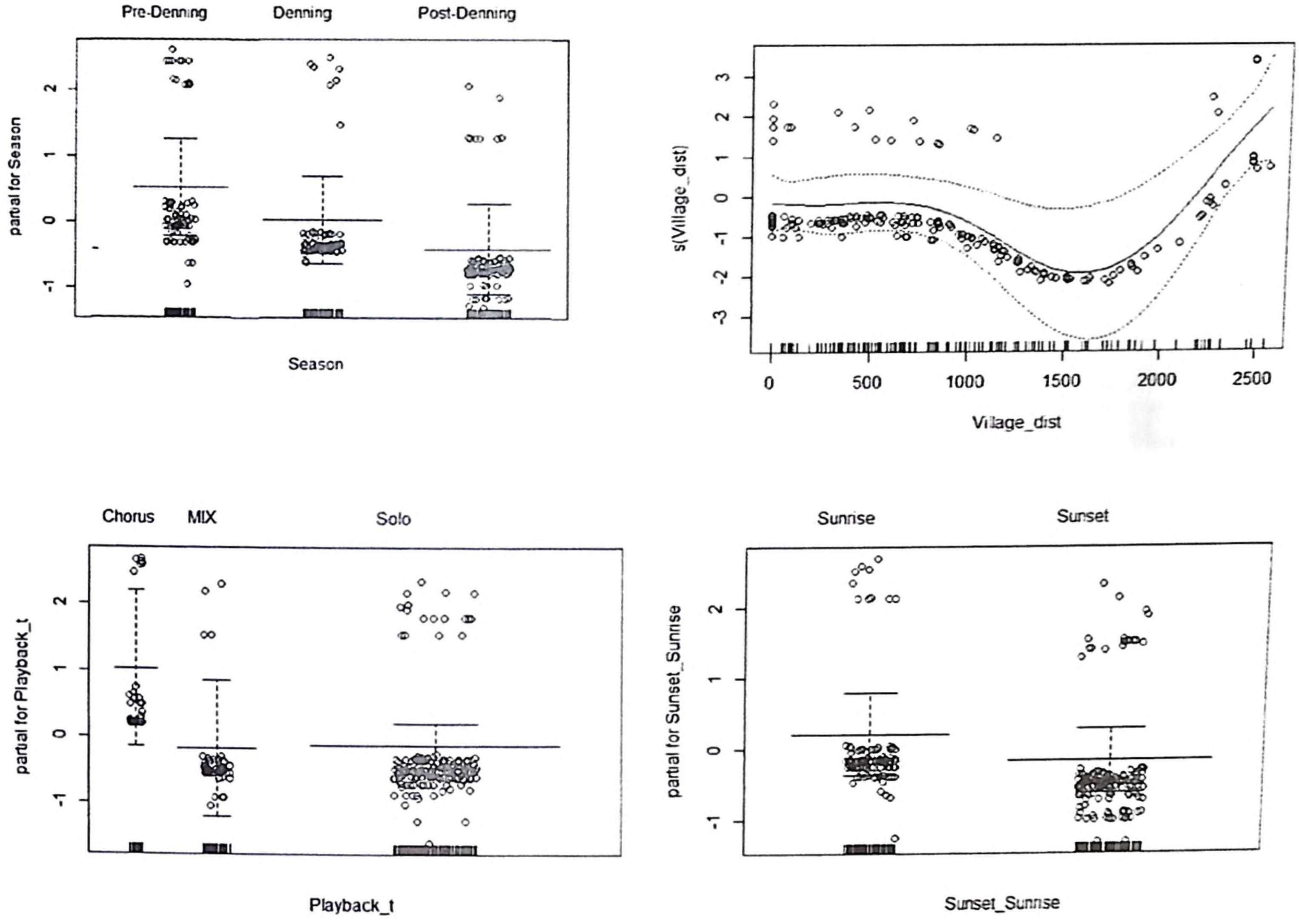

Figure 17. Graph showing how different factors influence howling responses in Indian wolf (non-collared) a) Wolves respond more during the pre-denning season compared to denning and pre-denning season. b) Wolf respond consistently from 0-1000 meters from villages and response rate increases after 2000 meters. c) Wolf responds more frequently to chorus playback. d) No significant difference in the howl response rate of the surveys conducted during sunset or sunrise.

EM and WM have varied human densities; therefore, I plotted the zone-wise (east zone and west zone) response rates to village distance [**Figure 18**]. In WM, the maximum response rate was obtained when surveys were conducted within 1200 meters from the villages (0.148 ± 0.031, n=128), which is significantly higher than EM (0.03 ± 0.021, n=66) (**Table 10; Figure 18**b). More importantly, wolves in WM showed a peak response rate in the HS 500-1000 meters from the nearest villages (RR=0.17 n=40) (**Figure 18**a). In comparison, only four responses were recorded in 57 howl surveys conducted at 1000-2500 meters from the nearest villages in the same landscape (RR= 0.07). In EM, wolves showed a high response rate in the howling survey 1200 metres away from villages (0.226 ± 0.075, n=31) (**Table 10; Figure 18**b).

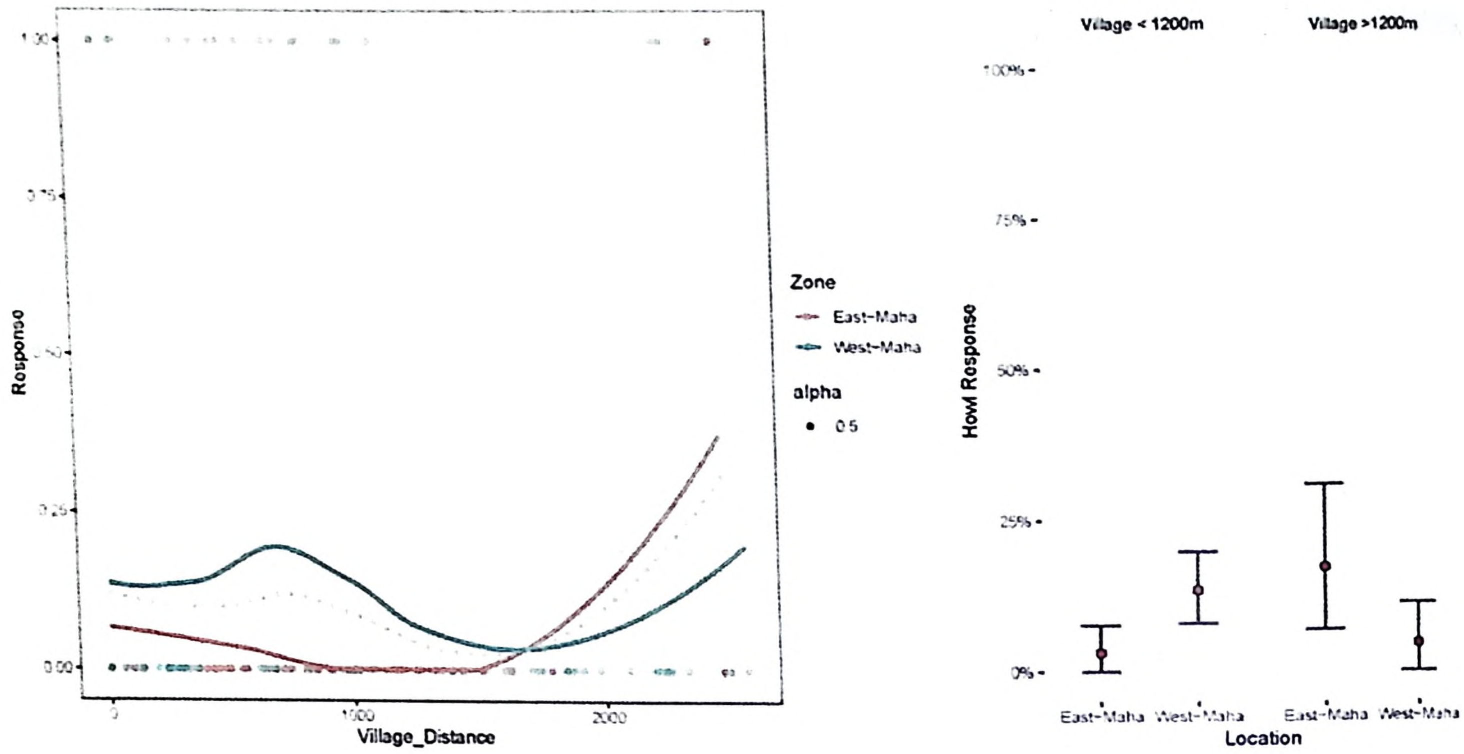

Figure 18. The graph shows how wolves from East (low human dense landscape) and West (high human dense landscape) Maharashtra have different tolerance levels toward village presence for howl response. a) Y-axis represents Response probability, while the X-axis represents village distance from the survey location. Each red and blue dot represents the howl response (0,1) of EM and WM, respectively. Howl response pattern with respect to village distance in EM, WM and combine is depicted through Red, Blue and dotted lines, respectively. b) Wolf response rate is significantly higher in WM (0.148 ± 0.031, n=128) than EM (0.03 ± 0.021, n=66) when howling surveys were done within 1200meter from the nearest villages. While the howling survey 1200meters away from the villages resulted in more response rate in EM (0.226 ± 0.075, n=31) than in WM (0.044 ± 0.03, n=31).

4.3.2 Factors affecting the howling responses of collared wolves

Due to anthropogenic land accusation pressure, most of the wolf packs were using fragmented home ranges with multiple core areas connected through villages or agricultural lands [**Figure 16**b] (Habib et al., 2021). The average core area of three adult wolves was found to be 35.0 ±17.94 km^2(N=8, Range: 0.68-29.32 km^2), whereas, for four subadults, the average core area was 3.1±2.81 km^2 (N=6, R: 0.46-2.44 km^2) [**Table 12**]. I also calculated the distance from the edge of each core area to the nearest village or town. It was found that the boundary of the core areas from the closest village boundary was 0-2500 meters.

Table 12. The home ranges and core areas of seven GPS-collared wolves in Maharashtra. The average home range of the Indian wolf is 168 km2, whereas the average core area is 117 km2.

Individual wolf	Area	95% BBMM (km^2) or Home range	50% BBMM (km^2) or Core Area
Breeze (M/A)	Gangewadi, Solapur	399.56	31.56
Merry (F/A)	Sangdari, Solapur	325.92	19.03
Firky (F/A)	Morgaon, Baramati	284.11	54.43
Finn (M/SA)	Ahmednagar	4.7	0.63
Rain (F/SA)	Ahmednagar	13.96	1.69
Rolfe (F/SA)	Saswad (Pune)	96.39	7.07
Rolf (M/SA)	Saswad (Pune)	54.35	3.11

M= Male; F=Female; A=Adult; SA=Subadult

I conducted 70 howl surveys from five packs across WM [**Figure 16**]. Wolf responded 39 times out of the total howling playbacks (n=70). As the presence of the collared wolf was confirmed using a VHF signal, the response rate was 56%. Like non-collared wolves, collared wolves also showed the peak response rate when the howling survey was conducted at 500-1000 meters from villages (RR=0.73, n=23). Through *'gam'* analysis I have found that animal's home-range ($F_{1,42}$ = 3.09, p < 0.001), maximum sound amplitude ($F_{2,42}$ = 7.43, p = 0.001), breeding season ($F_{2,42}$ = 4.73, p = 0.014) and animal distance ($F_{1,42}$ = 5.03, p = 0.03) are the significant factors influencing the howling response of Indian wolf [**Table 12**]. The wolves frequently responded when howl surveys were conducted within the core of their home range (50 % home-range contour), and the response rate dropped gradually away from the core area [**Figure 19**a]. Most of the time, the wolf responded in the second trial, which contains a playback with 75% sound amplitude of a 40w speaker [**Figure 19**c]. If they did not respond in the second trial, it was improbable to get a response from them at 100% amplitude of 40w Speaker [**Figure 19**c]. The breeding season also strongly impacted the howling responses of Indian wolves. Higher response rates were obtained during the pre-denning season compared to the denning and post-denning seasons [**Figure 19**b]. The ANOVA for smooth [s()] terms indicated significant non-linear relation between observer and respondent distance ($\chi^2_{3,42}$ = 7.68, p = 0.05) [**Table 13**]. Wolves frequently respond up to 1200 meters from the observer, but the response rate sharply drops after that [**Figure 19**b]. Although village distance shows a weak influence on the howling response ($F_{1,42}$= 1.85, p = 0.18) [**Table 12**], this is becaus the correlation between factors, as home-range cores are influenced by village distance [**Figure 19d**]. The response was maximum when the howl was played from 700-1100 meters from villages [**Figure 19d**]. However, our analysis did not incorporate anthropogenic disturbances in the surroundings during the survey.

Table 13. ANOVA table for Parametric Effects of probable influential factors on howl responses of Indian wolf (Collared). *The home range is the most critical factor that determines howl response (F value < 0.001), followed by the Maximum amplitude used in the playback survey (F values < 0.01). Other significant factors are Seasonality (based on breeding behaviour) and Animal Distance (F value < 0.05).*

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Significance Level
Season	2	5.4698	2.7349	4.733	0.014006	$F_{0.05}$
s(Animal_Distance)	1	2.9071	2.9071	5.0311	0.030231	$F_{0.05}$
s(Home_range)	1	7.7593	7.7593	13.4281	0.00069	$F_{0.001}$
s(Village_dist)	1	1.0703	1.0703	1.8523	0.180772	-
s(Sunset)	1	0.2329	0.2329	0.403	0.52897	-
Max_amp	2	8.5816	4.2908	7.4256	0.001733	$F_{0.01}$
Residuals	42	24.2694	0.5778			

Table 14. ANOVA table for Nonparametric Effects of the probable influential factor on howl responses of Indian wolf (Collared). *This table represents the significance level of smooth function, i.e., non-linear relation. The influence of animal distance and village distance is significantly non-linear with howl responses (F value < 0.1).*

	Df	Chisq	P(Chi)	Significance
s(Animal_Distance)	3	7.6885	0.052907	$F_{0.1}$
s(Home_range)	3	1.881	0.597414	-
s(Village_dist)	3	13.0046	0.004626	$F_{0.01}$
s(Sunset)	3	5.3356	0.148822	-

Similar to the non-collared wolves, the collared wolf data showed no significant effect of sunset or sunrise ($F_{1,42}$ = 0.40, p = 0.529) on howling response [**Table 12**].

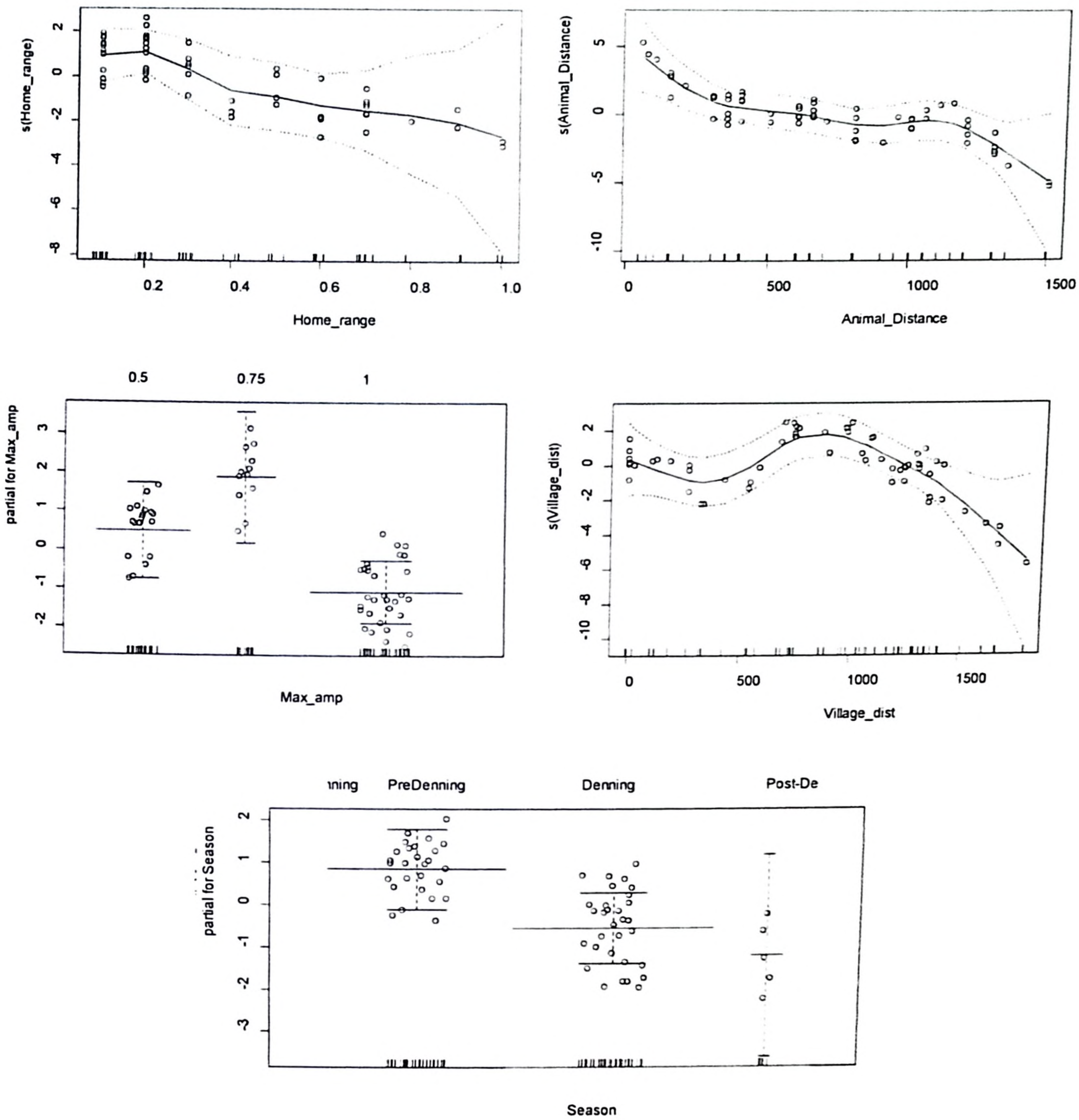

Figure 19. Graph showing how different factors cumulatively influence howling responses in Indian wolves (Collared). *Y-axis represents the howl response rate, and X-axis represents the corresponding factors. a) X-axis represents utilisation distribution distance of respective wolf pack (0.1-0.5 is core; 0.5-.95 is a home range). The response rate was higher in the core areas and decreased as the howling surveys were conducted away from the core. b) Rate of response gradually dropped as howling surveys were conducted away from the collared animals. Animal distance might affect the observer's detection of response due to high anthropogenic noise in the human-dominated landscape. c) Graph represents wolves primarily responding on the 2nd trial, which uses 30w playback sound (0.75 sound pressure of 40w speaker). It's unlikely to get a response in the playback that contains 40w sound pressure. d) figure illustrating how distance from the nearest village influences howl response. Wolf responded more frequently during the howling survey conducted 700-1100 meters from the villages. e) graph showing howl seasonality (based on breeding behaviour) affected howl response rate. Wolf responded more during the pre-denning season, and the response rate dropped during the denning and post-denning seasons.*

4.4 *Discussion*

The indirect evidence of top predators through scent marks or vocalisation shapes domestic and wild prey's behaviour and active foraging time (Cooke et al., 2013; Janczarek et al., 2021). Therefore, the presence of wolves in an ecosystem has both direct and indirect impacts on the tropical cascade. Our study was focused on the long-distance vocalisation of Indian wolves in two habitats with varied human disturbance through the howling survey. I obtained dissimilar response rates depending upon the core-home range and village density. In the low disturbed EM, wolves mostly avoid responding to howl surveys if done within 1500 meters from villages. In high human dense WM, they showed the highest response rate 500-1000 meters from the villages. Although the study on collared wolves revealed that their home range cores areas govern their response rate, they are less likely to respond in the core areas adjacent to the village boundary. The wolf might avoid howling in those landscapes to prevent ease of detection by barring from howl response. Therefore, to survive in high human-altered habitat, wolves might need to sacrifice their fundamental territorial vocalisation behaviour. Although conservationists are primarily concerned about the extinction of species, this study highlights the vulnerability of fundamental behaviour of a keystone species attributed to human-induced contemporary evolution. Howling is a critical behaviour of this pack living species, which determines their reproductive success, social cohesion and pack bonding. Therefore, this fundamental behavioural change can alter the biology of the keystone species to impact the ecological cascade.

4.5 *Home-range and Anthropogenic factors influencing the howling response of Indian wolves*

Scientists have previously emphasised how factors such as pack dynamics, home range, time of day, and seasonality influence howl responses, keeping anthropogenic factors unnoticed. This study measures the impact of village distance on howl response with the combinations of earlier established factors to avoid any possible biases. The howling survey was conducted on both the non-collared and collared wolves. I found that the wolf's home range primarily governs the howl response rate followed by village distance. The response rate is almost double in the respective core areas ($P_{0.63}$; 33 responses on 52 occasions) than in the buffer ($P_{0.33}$). The pattern is similar to what Harrington and Mech (Harrington & Mech, 1978a) reported in their study ($P_{0.29}$ in the non-rendezvous site and $P_{0.78}$ in rendezvous sites). Collared wolf data also reveals that home-range cores are restricted mostly by village and agricultural patches. In *'gam'* analysis, I observed wolves were more responsive at 700-1100 meters from nearest villages, and RR further increased from 2000meter from villages after a certain dip at 1500meter [**Figure 17**b]. To understand the reason behind the drop, I plotted EM and WM's howling response rates distinctly; it showed a clear distinction in the howling response pattern between the two zones [**Figure 18**].

In the EM region exhibiting less village density, wolf almost does not respond to howl playback up to 1500meters from villages and the response rate increases after that. Whereas in human-dominated WM, wolves exhibit greater tolerance towards villages. Wolves showed a high response rate to howling playback 700 meters from villages because, in a human-dominated landscape, they are highly adapted to using village resources (Habib, 2007; Jethva & Jhala, 2004b; Singh & Kumara, 2006). The howling surveys that were done 1200 meters away from villages observed a drop in the howling response rate of WM. As WM is densely packed with villages, once wolves move away from one village, the chances are higher that they will find another nearby village. In WM, due to high village density, the wolf has the less free habitat to move, whereas in EM, with less density of village, wolves have more habitat space to move, showing no inclining after the response rate peak [**Figure 18**]. Although the datasets are independent, the howling survey on collared wolves (WM) showed a similar pattern (Figure 5d). Even if some of their home-range cores were situated adjacent to the villages, they rarely responded to a howl playback once the howling survey was conducted closer than 700 meters from a village. Since howling very close to villages might increase their detection and vulnerability towards humans, they restrict themselves from responding to howling playback in those areas. In the course of development, the villages may expand further, which might leave far less habitat for wolves to defend by their long-ranging howl. Therefore, wolves might need to conceal their presence by avoiding the howl to survive in the human-dominated landscape soon. Howl is also a mode of territorial advertisement to avoid inter-pack antagonism (Harrington & Mech, 1978a). Without howl and high resource competition in a human-modified landscape, the wolves may face a high degree of physical conflict with neighbouring packs. The possibility of wolves losing a 'Landscape of fear' can not be ignored as wolf howl has a significant impact on prey, co-predator, and domestic animals' fear and their foraging behaviour (Cooke et al., 2013; Janczarek et al., 2021; Suraci et al., 2016).

4.6 *Standardising protocols for howling survey in a human-dominated landscape*

For the conservation of wolves, I needed an efficient technique to study their population and biology. Studies suggest that the howling survey is the potential and most efficient non-invasive technique for studying the cryptic wolf (Garland et al., 2020; Harrington & Mech, 1982; Suter et al., 2016). I have found that the breeding season strongly influences the howl response rates, and wolves responded more to the howl playback during the pre-denning season than during the denning and post-denning seasons. Higher response rates support that wolves hold their territories antagonistically in the pre-denning season. The response becomes restricted from denning season onward since howling from the den or near the early-aged pups might make pups vulnerable to the invaders. A study on the Yellowstone wolf also suggested that wolves respond more frequently during their breeding season, i.e. February (McIntyre et al., 2017), similar to the Indian wolf. However, the breeding season for Indian wolves (pre-denning season in our classification) is around early November. Therefore, the preliminary information on wolf breeding behaviour will facilitate an efficient howling survey design. In contrast, the study in North-Eastern Minnesota National Park (Harrington & Mech, 1982) and Białowieża Primeval Forest (Nowak et al., 2007) revealed a second peak in the howl response rate from July to September [post-denning season]. I did not observe such a trend in Indian wolves because the post-denning season overlaps with the Indian monsoon, and no howling survey was conducted during that period. Besides those behavioural (breeding season and home range) and anthropogenic factors, the response rate also depends on a few stimuli factors such as distance, type, and intensity of the playback. I found that wolves respond more frequently towards chorus howls. Since, many times in the field, wolves visited the sound source after hearing a solo howl instead of responding (field observation), I nullified the possibility of the animal missing the solo howl, due to a higher attenuation rate. They might prefer howling in response to the chorus for defending their territory and avoid direct visit toward the visitor pack at first to steer away from physical conflict. For solo howl they have less threat from individual and they prefer direct visit. Further investigation is required to understand this behaviour. The data from collared animals revealed that animal response rate drops when they are more than 1200 meters away from the playback source, or it might be the chance of detecting the response decrease from the observer end due to anthropogenic noise. Collared wolves responded mostly in the second trial (if not in the first trial that uses 50% sound amplitude) with 75% sound amplitude of 40w speakers. If they do not respond to the second trial, the chances of getting a response are unlikely in third (with 100% sound amplitude) or consecutive trials. It can be concluded that a 30w speaker (75% amplitude of 40w speaker) is sufficient for doing a howling survey which is loud enough so that wolf can hear it from 1200-1500 meters in the high human disturbed landscape, but not as loud as to restrict the wolf from responding. Since I can detect a wolf through its howl from 1.2 km in either direction, the optimal grid size for doing systemic howling survey in a human-dominated landscape is 1.7×1.7 km^2 [Calculated from the formula of square inscribed in a circle, $1.2 \times \sqrt{2} = 1.69$]. The animal distance graph from the collared wolf will help in calculating the detection function for population estimation model through the howling survey.

4.7 Limitations of the present study and future directions

I address many critical aspects of wolf conservation in our study. The wolf collaring program was conducted exclusively in WM because of resource limitations. A comparative study on the collared wolf from EM would have been a more substantial way to conclude our findings. Although I have found that wolves visit survey locations more often as a response to solo howls from our field observations, the quantitative data is not available to test its significance level. The noise level would be a piece of additional information that might influence the detection of animal response. However, I was not able to include the impact of noise due to sample size restriction.

4.8 Managemental recommendation

Ecologists are often delighted to see human-animal coexistence and are more concerned about how adaptation in the human-modified environment saves them from extinction (Gross et al., 2021; Madden, 2004; Pooley et al., 2021). Human-induced adaptation habitually alters the fundamental behaviour of many species. A few studies explored the aftermath of this behavioural alteration that shifts the top predator's ecological role. The crucial finding from our research shows the potential adaptation in the fundamental howling behaviour of the Indian wolf in response to the high human-dominated landscape, which may critically impact the whole ecosystem. Therefore, surviving in mosaic patches of the human-modified landscape may save the wolf from local extirpation, but the impact on their long-range signatory call might have a severe outcome for the species and the landscape. Ecologists often solely consider the physical space in their conservation efforts, but our study highlighted that the acoustic space critically influences the fundamental behaviour of the vocal species. As the vocalisa is associated with reproductive success and social cohesion of this pack living species and impacts the foraging behaviour of the lower cascade, an urgent conservation effort is required so that 'acoustics space' is not compromised. Howling without increasing vulnerabilities is possible only in continuous large wolf habitats instead of conserving them in habitat fragments of a human-altered landscape. These required a national park-centric species conservation approach for wolves. This requires a significant policy revision in India to protect grassland habitats for conserving wolves. This national-level conservation plan might provide the necessary acoustics space for the wolves, which is the only way to save the keystone species with their fundamental behaviour and functional ecological role.

CHAPTER 5 Towards a reliable population estimation of Indian wolves using a non-invasive howl survey

5.1 Introduction

The Indian wolf (*Canis lupus pallipes*) is considered an *Evolutionary Significant Unit* (ESU) due to their close genetic to the ancient wolf population (Hennelly et al., 2021). Indian wolves have a wide distribution range in the subcontinent - latitudinally extending from Rajasthan to Karnataka and longitudinally from Gujarat to West Bengal (Gubbi et al., 2020; Jhala & Giles, 1991; Saren et al., 2019; L. K. Sharma et al., 2019). They are primarily found in non-protected areas, mainly in village outskirts (Habib & Kumar, 2007; Jhala & Giles, 1991; L. K. Sharma et al., 2019; Singh & Kumara, 2006). As Indian wolves mostly survive in the human-dominated landscapes and a significant portion of their diet is domestic livestock (Habib & Kumar, 2007; Jethva & Jhala, 2004b; Singh & Kumara, 2006), they are subjected to human-animal conflict in the landscape (Agarwala et al., 2010). Despite their evolutionary importance and threats to their population, the population status of the Indian wolf is poorly known.

The conservation status of any species depends on its population size; as smaller the population, the more vulnerable it is to extinction (Mace et al., 2008). The knowledge of species presence and abundance is critical for informed management decisions, especially for a species like the Indian wolf, which is under threat in human-dominated landscapes. It shows the importance of population estimation of Indian wolves at regular intervals. However, population estimation of the wolf has been a worldwide challenge for decades (Blanco & Cortés, 2012; Garland et al., 2020). Wolves actively avoid camera traps (Meek et al., 2014), and due to an extensive home range (Habib, 2007; Habib et al., 2021), visual encounter-based models such as transect sampling fall short in estimating their population density (Stenlund, 1955). Although they are visually cryptic, wolves can be detected from very long distances through their long-ranged vocalisation, i.e. howl (Suter et al., 2016). As the howl is their territorial call, wolves respond to howl playback to defend their territories (Harrington & Mech, 1978b; Joslin, 1966). The concept of wolf census using playback is long-standing (Harrington & Mech, 1982). Still, some technical and statistical limitations restricted the use of howl surveys in the population estimation of wolves for years.

5.1.1 Literature Review and available methods for population estimation of wolves

Wolves exist in low numbers in large areas, and their cryptic behaviour makes it extremely difficult to estimate their population (Linnell et al., 1988). Snow-tracking is used for estimating the wolf population in European and Northern American countries (Kojola et al., 2014). But this study only reports the number of minimum reproductive units due to high error values and the difficulties of tracking solo wolves using snow-tracking (Kojola et al., 2014). More importantly, snow-tracking is not a feasible technique for most wolf countries. The population trend of wolves was previously assessed using open-model capture-recapture (Marucco et al., 2009). In recent years, Wolf Population estimation was done using DNA monitoring (scats) combined with the Spatial Capture-Recapture Poisson approach (López-Bao et al., 2018). The critical challenge in DNA sampling is variation in individual wolves' scent-marking patterns, resulting in biased estimation (López-Bao et al., 2018; Marucco et al., 2009). Young lone individuals deposit their scat off-trail to hide their presence, making it extremely difficult to collect their scats (Rothman & Mech, 1979). In the era of rapid technological growth, bioacoustics surveys have been popularised for visually elusive but vocally active species worldwide. Previously, the Howl-box (self-contained broadcasting device for simulated howls and recording howls) was developed to monitor the presence of the wolf, but it resulted in a very low detection zone due to the omnidirectionality of the microphone (Brennan et al., 2013). In recent years, Passive Acoustics Monitoring (PAM) has been tested for wolf monitoring in several parts of the world (Garland et al., 2020; Rhinehart et al., 2020; B. Smith et al., 2021; Suter et al., 2016). Calculating animals' location from their call is vital for calculating the detection function in density estimation (Marques et al., 2013). In PAM, the animal locations were calculated from multiple arrays of Automated Recording Units (ARU) (Garland et al., 2020; Papin et al., 2018; Stevenson et al., 2015). The main challenge of population estimation using PAM is calculating the call rate in a natural scenario (Marques et al., 2013). Therefore species density estimation was never done using PAM. Moreover, commercial ARUs are costly, and low-cost ARUs compromise the smaller detection zone – low-cost ARU are 40% less effective than commercial devices (B. Smith et al., 2021).

Wolf howl can be detected from a considerable distance in manual surveys (Harrington & Mech, 1982; O'Gara et al., 2020), and high-quality recording can be yielded using a unidirectional microphone (Font et al., 2015; Palacios et al., 2007, 2016). Active howl surveys are a cost-effective tool for surveying wolves — a high-end unidirectional recording-set costs around the same as a single commercial ARU. Since the main challenge of an active howl survey is to calculate the animal distance from their response, in this study, I have focused on determining the distance of respondent animals through the multiple observer method. The fundamental goal of the study is to standardise an active howl survey-based population estimation tool for the Indian wolf. By delineating the wolf habitat in four districts of Maharashtra, I conducted a pilot study to estimate the population density of wolves. With the ability to estimate the population density of wolves through an active bioacoustics survey, we can build a cost-effective tool to assess a landscape based on its apex predator status. The method can provide a guideline to estimate the wolf population worldwide.

5.2 *Material and Methods*

5.2.1 *Study Area*

The study was conducted in the semi-arid drought-prone areas of the Deccan peninsula Biogeographic Zone in Maharashtra, India (Zone 6) (Rodgers & Panwar, 1988). Deccan thorn scrub forests are the dominant habitat type in the sampling areas (Reddy et al., 2015). The main characteristics of the Deccan peninsula are mild undulating slopes and flat-topped hillocks with intermittent shallow valleys. Wolves were primarily found in grassland and thorn scrub forests, highly fragmented by agricultural lands and small villages.

5.2.2 *Identification of the wolf habitat*

I conducted a reconnaissance survey to identify the wolf habitat by interviewing local shepherds, followed by a scat collection and howl survey from December 2015 to July 2016. Only reliable scats were collected as wolf and dog scats are challenging to distinguish. The reliability of scat was ensured by its contents (mostly hair, bone and seeds of *Ziziphus sp.*) and its strategic locations. As scat plays a crucial role in territory marking, wolves deposit them in strategic locations such as crossroads or surfaces above ground (Barja et al., 2004). The detailed howl survey methodology was published by Sadhukhan et al. (Sadhukhan et al., 2019, 2021).

Five wolves were captured using soft-catch leghold traps and were fitted with satellite radio collars. The detailed methodology was published by Habib et al. (2021). The GPS fixes of collared wolves from December 2017 to October 2019 were used to model the habitat using Maxent analysis.

5.2.3 Delineating wolf habitat

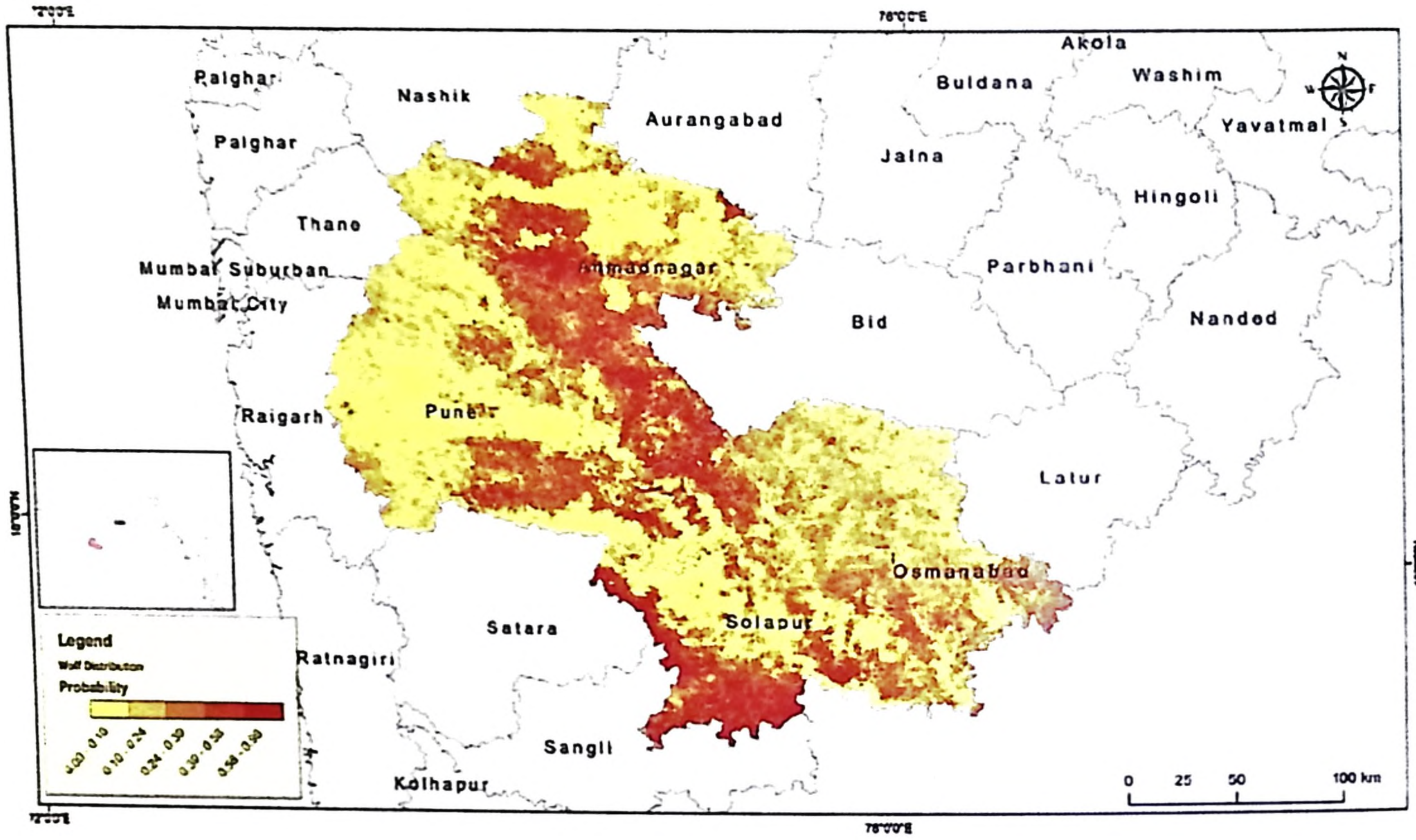

Figure 20. Map showing the probability of wolf distribution in four districts of Maharashtra. Red Represents high distribution probability, and yellow stands for the less probable area for wolf distribution

Maximum Entropy Probably Distribution (Maxent) was used for delineating the potential wolf habitat in four districts of Maharashtra – Ahmednagar, Pune, Solapur and Osmanabad (**Figure 20**)(Phillips et al., 2017). The locations of scats, direct sighting and collared wolf data were used as the presence location of the wolf. Spatial filtering was applied in a 1086×1086-meter grid to maintain uniformity in the presence location. A total of 147 locations were used with a bias layer. The bias layer defines the sampling area to avoid underprediction and clustering of ecological data output (Syfert et al., 2013). The average NDVI, nightlight, ruggedness, precipitation during the wettest quarter, and the Euclidean distance from the water body, building, grassland, plantation, fallow-land, Ravi crop-land, and Kharif crop-land were used as variables to the maxent model. Each variable was uniformly resolute to 1086×1086 meter cell size (based on the smallest cell size among all the variables).

The maxent model was trained using 75% the wolf presence locations with eleven mentioned variables and 25% presence locations were used test the model. Fifteen replicative runs were performed to assess the variability in the model prediction. Since the model gave output as distribution probability the effective wolf habitat were calculated by multiplying the probability value with the grid size.

The probable wolf distribution map from maxent analysis was further categorised into highly suitable (0.58-0.98), suitable (0.24-0.58), and low/non-suitable (<0.24) areas based on the probability value of each pixel. A 1500-meter buffer was added in the highly suitable and suitable area, and then it was cropped using nightlight to avoid sampling in cities, towns or villages. A fishnet was placed with a grid size of 1.7 × 1.7 km^2.

5.2.4 Study Design

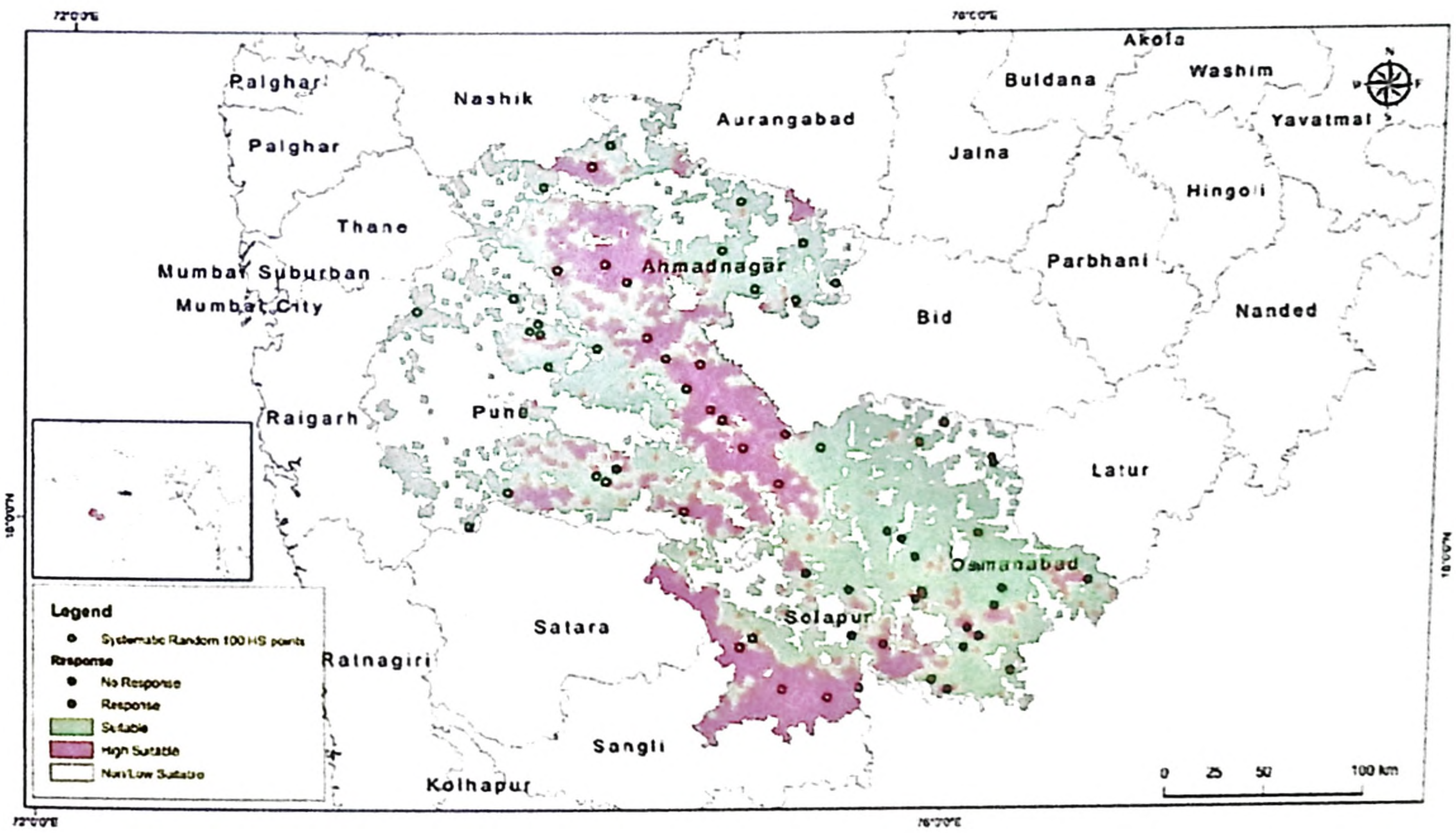

Figure 21. The map represents the highly suitable (pink) and suitable (green) areas of Indian wolves in four districts of Maharashtra. Green dots represent 100 systemic grid random points for conducting the howling survey exercise. The blue dot represents no response, and the red dot represents the howls response location.

One hundred random grids were selected from the study area fishnet grids (1.7 × 1.7 km^2=2.89 km^2) to conduct a howl survey for the population estimation of Indian wolves (**Figure 21**). The criteria of grid size were discussed in Section 4.4 (Para 3) of Chapter 4. During the howling survey, three observers were positioned 150-500 meters away from each other within the selected grid (**Figure 22**). Observer 1 (O_1) played the pre-recorded howls for the survey. Observer 2 (O_2) and Observer 3 (O_3) recorded the bearing playback alongside GPS location. This playback bearing (PB) helps to assess the bearing accuracy. All the observers took the bearing of a respondent wolf if they detected the howl response. The bearing of the respondent wolf from at least two observers is required to detect the wolf's position. The position would help calculate the distance and determine the detection function.

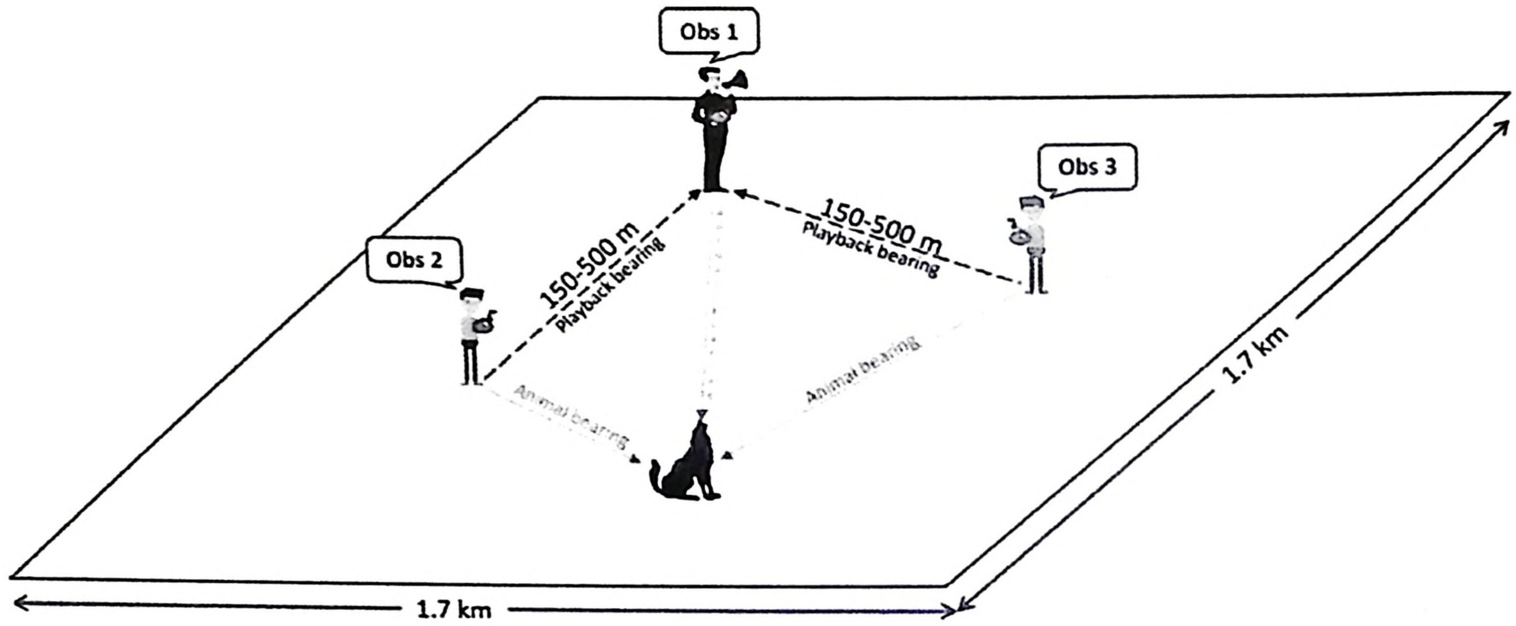

Figure 22. The illustration describes the howling survey design based on a triple observer with a grid size of 1.7×1.7 km². Observer 1 plays the pre-recorded chorus howl. Observer 2 and Observer 3, stationed at 150-500meter apart from O_1, record the playback bearing. Once the observers detect the howl response, they record the bearings.

5.2.5 *Measurement of the bearing accuracy*

Twenty-five grids were sampled using howling surveys out of 100 randomly selected grids. The actual bearings and the distance from O2 to O1 and O3 to O1 were calculated from their GPS coordinates, and linear regression was executed to test the accuracy of Playback Bearing (PB) with observer distance (**Figure 23**). The distance between O1 and O3 was estimated (maximum and minimum) using Equation 2 to compare it with the original location of O_1. The minimum and maximum possible distance between O_1 and O_2 were calculated similarly.

$$Distance\ b/w\ O_1\ and\ O_3 = \frac{Distance\ b/w\ O_2\ \&\ O_3\ \times sin(\angle O_3 O_2 O_1)}{sin(\angle O_2 O_1 O_3)}$$

$$= \frac{Distance\ b/w\ O_2\ \&\ O_3\ \times sin(PB\ O_2 - CB\ O_2\ to\ O_3)}{sin\{(PB\ O_2 + 180) - (PB\ O_3 + 180)\}}$$

$$= \frac{Distance\ b/w\ O_2\ \&\ O_3\ \times sin(PB\ O_2 \pm BE - CB\ O_2\ to\ O_3)}{sin(PB\ O_2 \pm BE - PB\ O_3 \pm BE)}$$

Equation 2

**PB = Playback Bearing; CB = Calculated Bearing from GPS locations; BE=Bearing Error*

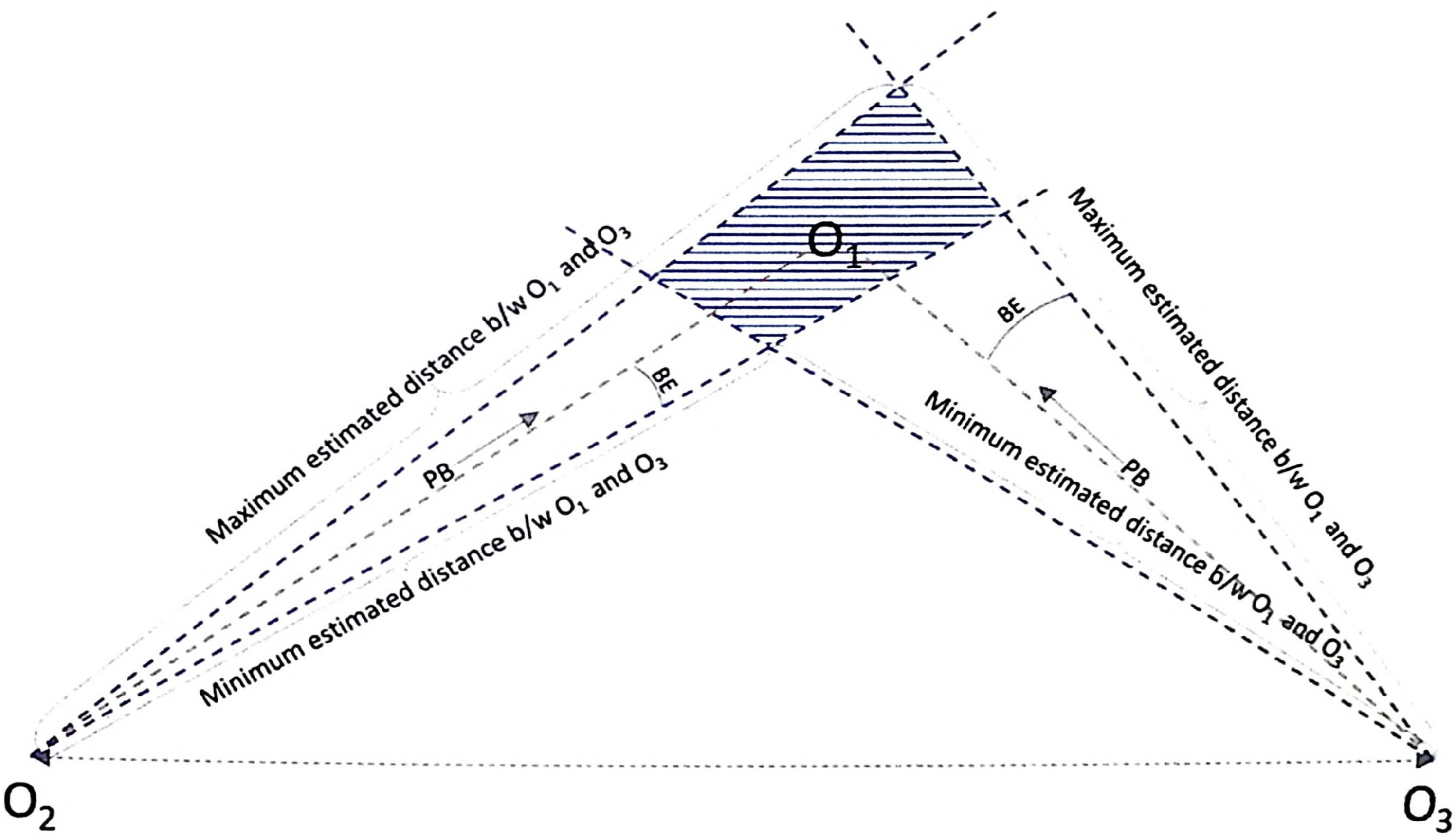

Figure 23. ***The graph shows the technique to estimate the location of Observer 1 from the GPS position and bearing of*** O_2 ***and*** O_3*. The Orange dotted line represents the actual bearing, and the blue dotted line represents the estimated position range of* O_1 *considering the bearing error.*

5.2.6 *Population Density Estimation using 'ascr' (Acoustics Spatial Capture-Recapture)*

The data obtained from the howling survey were analysed with package *'ascr'* in R (v 4.1) to estimate the population of Indian wolves. The *'ascr'* package is based on Spatial Capture-Recapture (SCR) model to estimate animal density from acoustics surveys (Kidney et al., 2016; Stevenson et al., 2021). Each of the observer locations was considered a trap location; therefore, 75 trap locations were used as a trap matrix (3 observers × 25 Survey sessions). The howl response detected by an observer was considered as captured at a specific trap. Once two or more observers detected the responses in a single survey session, it was recognised as a recapture. The bearing from two or more observers helps the model calculate the animal's distance, detected through howl response. A 1500-meter buffer was used as a mask based on the data from the howling survey of the collared wolves (See 4.3.2 in Chapter 4). A mask defines the maximum detection zone of howl response from an observer. From the study on collared wolves, I found wolves responded 56% of the time (n=70), and the response rate was 70% once the howling survey was done with chorus howl in pre-denning season (n=21). When the animal is closer than 500 meter, the response rate is 0.83% (See 4.3.2 in Chapter 4). The minimum g_0 or the chance of getting a response when the wolf is a zero distance is 0.83, considering the entire exercise were done using chorus howl in pre-denning season. The 'ascr' analysis was done with three different g_0 values – 0.8, 0.9 and 1. In the SCR model, sigma is a crucial factor for population estimation, representing a detection function related to how far the animal ranges during the survey (Royle et al., 2013; Sun et al., 2014). Whereas, in the Acoustics SCR model, the detection function relates the distance between the physical location of the respondent (here wolf) and an acoustic detector (Observer) (Royle, 2018; Stevenson et al., 2015). Therefore, the sigma in 'ascr' represents how far the sound travels at a detectable level. As the 'effective sigma' of the encounter probability were unknown, the analysis was done with different sigma value (200-1500) to find the best suitable model. my response sites were very far from each other (Closest response sites were 25km apart surveyed in the same day an hour apart, whereas other response sites were minimum of 30 km away from each other). I assigned unique individual IDs to each of the respondent animals.

5.3 *Results*

5.3.1 *Delineating wolf habitat*

Eleven variables were used to delineate the potential wolf habitats in four districts of Maharashtra (total area = 54607 km^2) through maxent analysis (**Figure 20**). The average AUC value for testing the model was 0.545, with a standard deviation of 0.031. Average NDVI (19.2%), Euclidean distance of Ravi croplands (11.1%), Euclidean distance from grassland (10.3%), precipitation of wettest quarter (10%) and nightlight (9.6%) are the most important contributing factor in the model. The total effective wolf habitat found thru the model was 12250 km^2.

5.3.2 *Howling survey and its bearing accuracy*

Out of 100 targeted howling surveys, I conducted 25 surveys due to logistical constraints (**Figure 21**). Howling responses were obtained in seven locations. Out of 25 howling surveys, O_2 detected 24 playbacks compared to O_3 successfully detected 23 playbacks. The bearing error was calculated from the observer location and PB. The average bearing error by O_2 and O_3 was 7.95° and 11.95°, respectively. No significant relation was observed through linear logistic regression between the observer distance and the bearing error (**Figure 24**). After estimating the location of O_1 from average bearing error, I have found that 20 times (n=23), the actual location of O_1 was within the range of the estimated location. The average estimated distance range (difference between the maximum and minimum distance) of O_3 to O_1 was 216.67 (±90.17) meters.

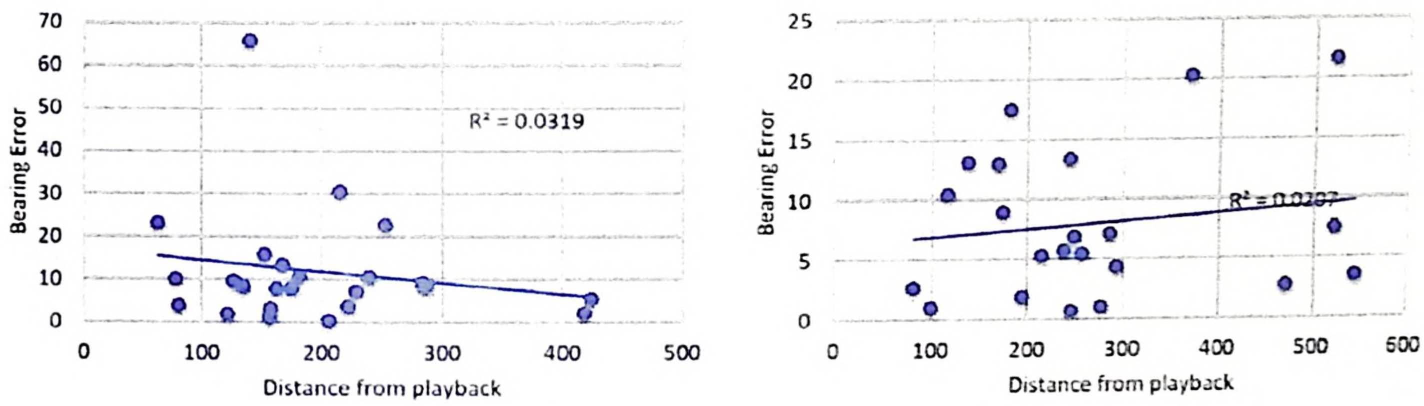

Figure 24. Linear regression between the distance of playback bearing and the bearing accuracy. The left side graph represents PB that O2 took, and the right graph represents PB from O_3

5.3.3 *Population Density Estimation using 'ascr'.*

I detected seven individuals through the howling survey with three recaptures (**Figure 25**). The ten spatial captures from 75 trap locations through 25 active howling survey sessions were analysed through *'ascr'*. The analysis was performed with different g_0 and sigma values. The best-fitted model has a sigma value of 1200, and the error rate is also consistent. The sigma value of 1200 (the distance sound travels at a detectable level) is comparable with the howling survey done on collared wolves (See **Figure 19b** in Chapter 4). There were no significant differences within the error range of different g_0. Therefore, the population density of wolves is 3.65 individuals/100 km^2 with a lower limit of 1.67 to an upper limit of 5.63 (95% CI) [g_0=0.9] (**Figure 26**). Therefore, the total wolf population in the habitat was a minimum of 205 to a maximum of 690 (95% CI).

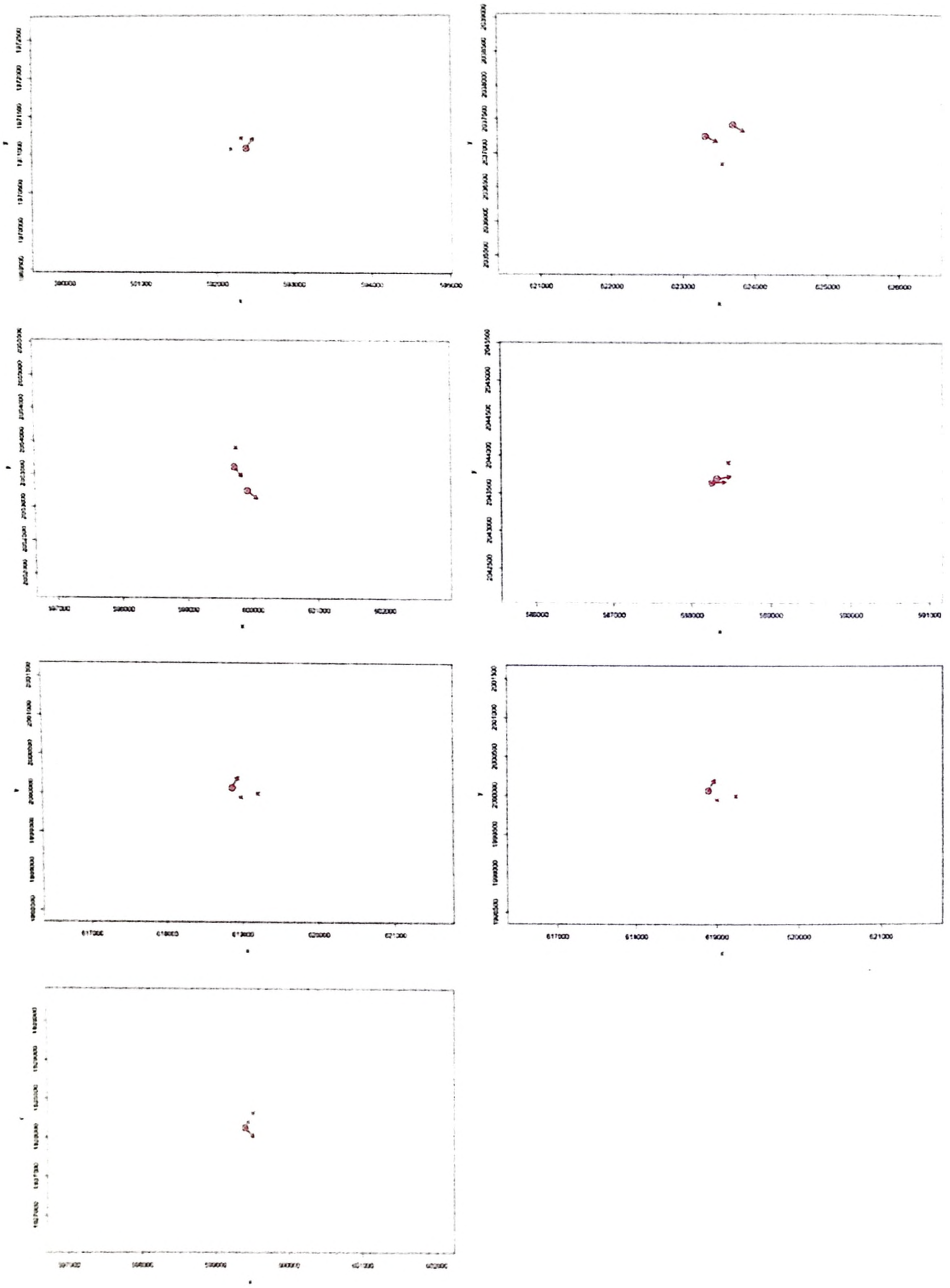

Figure 25. Each graph represents each of the seven survey sessions with the location of three observers. *The red arrows direct (bearing from the observer) toward the animal's location. In diagram 1, only one observer detected the call. In comparison, in diagram 2, two observers detected and recorded the bearing of the responded animal.*

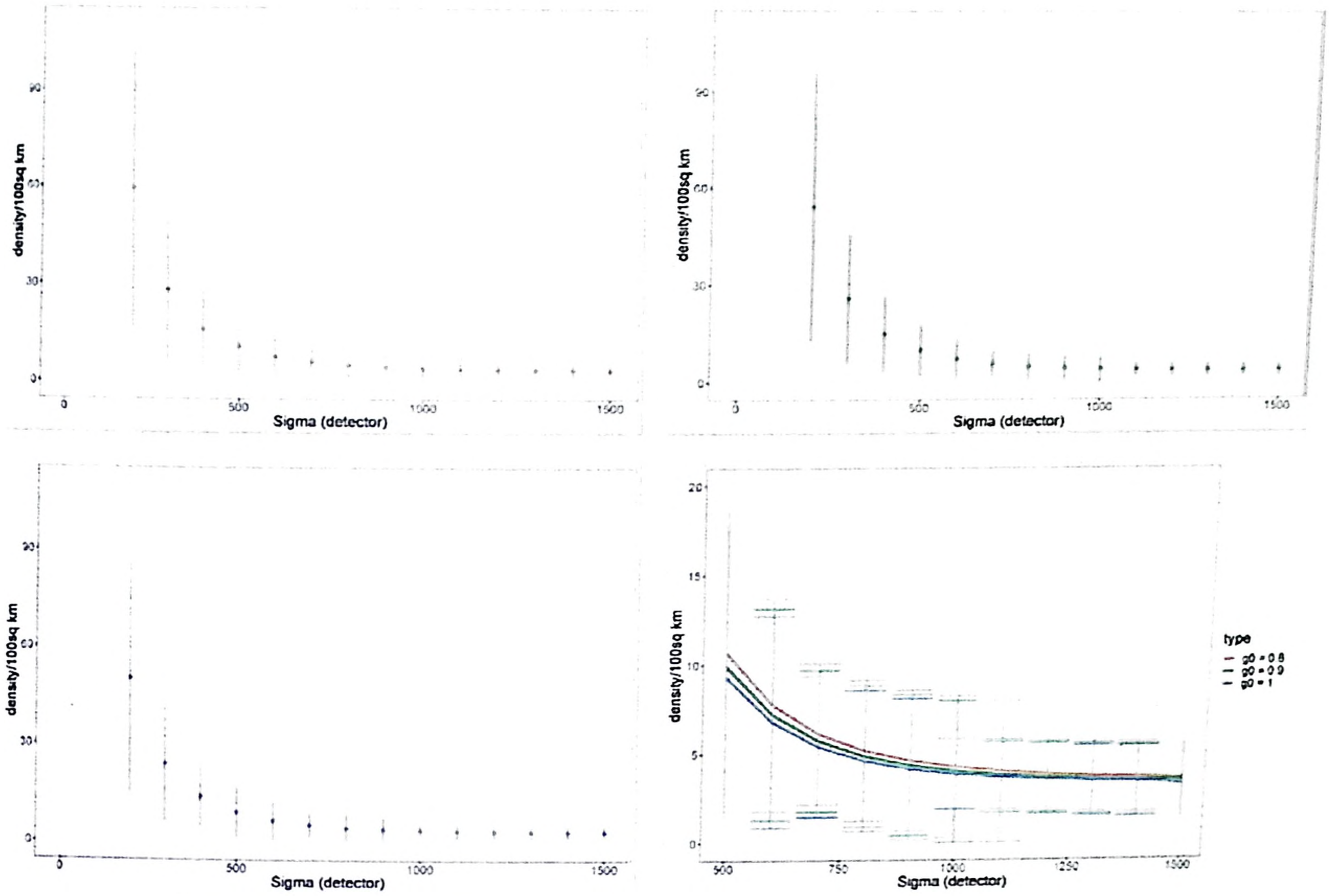

Figure 26. Each graph represents the estimated density (y-axis) and value of sigma (x-axis) with respect to the value of g0. g0=0.8, g0= 0.9 and g0=1 are represented by red, green and blue, respectively, with the error bar. Asymptote reached a sigma value of 1200, and the error is very consistent after the value. The density of wolves is 3.5 (=1).

5.4 Discussion

Here I presented a newly adapted triple observer-based active howling survey method to estimate the population density of wolves. Bearing from three observers, popularly known as the triangulation method, was used to calculate the distance of the respondent animal. The prime challenge in acoustics-based population estimation is to calculate the distance of respondent animals to generate a detection function. PAM devices overcame the issue with the array of ARUs, whereas the challenge remained unaddressed for manual active surveys. I overcame the concern using observer bearing, making the method very cost-effective. The key to concern in PAM is determining the call rate, which is not applicable over the active howl survey. However, an active howl survey requires information on g_0 or probable response rate of wolves at zero distance. The minimum g_0 was calculated from the howling survey data of collared wolf, and I did not find any significant differences between the density estimation of minimum and maximum g_0. This study has developed a methodology for delineating the wolf habitat and the protocol for doing a howling survey for population estimation of the wolf. The study's key finding is the population density estimation using a playback survey, where I have found 3.65 (±1) individuals/100 km^2 area. This is the first successful attempt to estimate the population density of Indian wolves through a non-invasive active acoustics survey. A systematic howl survey in the subcontinent will reveal the actual population status of the Indian wolf, which will play a significant role in the conservation of the species, which is recognised ESU. Since the global wolf population has remained unknown due to the lack of a standardised method, the protocol from my study will provide crucial guidance in estimating wolf density around the world.

The primary challenge was delineating the wolf habitat in a human-dominated landscape to estimate the density. I used the standard maxent distribution model to find the habitats. The model provides the distribution probability. Thus, as for the basic assumption of the probability theory, if 100 grids were sampled with a 70% probability of distribution, 70 grids are likely to have wolf distribution if the model is correct. I used the same principle to find the total effective wolf habitat in four districts of Maharashtra. With systematic sampling thru a larger sample size, the methodology might ground-validate the probability distribution model from the howling survey data. My pilot survey found 3.65 individuals/100 km2 with a lower limit of 1.67 to an upper limit of 5.63 (95% CI) in the four districts of Maharashtra. Since no statistical estimation is available for Indian wolves, I do not have a point to compare my data. However, the density range and error rate overlap with the density estimated using DNA sampling by López-Bao et al. (2018) for the Iberian wolf population [2.55 wolves/100 km2 (95% CI = 1.87–3.51)].

The main advantage of using active howling survey methods for density estimation is cost-effectiveness. The study requires three observers with compasses, a playback speaker and an optional directional microphone. The recording will help in additional information like individual identification. In contrast, ARU-based PAM might require less workforce during sampling, but howl detection and data extraction require heavy computational power with a labour-intensive skilled workforce. Moreover, commercial ARUs are costly, allowing limited area sampling, and the low-cost ARU has a small detection zone (B. Smith et al., 2021). Comparatively, a manual active howl survey long gun microphone gives clearer recording and a wider detection zone—a recording set with a long-gun microphone cost as same as a single commercial ARU. Moreover, the manual active howling survey is widespread in many countries to know the presence of wolves and their reproductive status (Gable et al., 2018).

Therefore, the methodology can be implemented in many parts of the world for the population estimation of wolves without any significant up-gradation in equipment.

For any acoustics survey, the prime challenge is calculating the animal distance for the detection function. The animal distance can be calculated from the bearing, but the bearing comes with some error which varies with different factors such as wind speed or terrain (Gable et al., 2018; O'Gara et al., 2020). Without placing the bearing error in the model, it might lead to an erroneous prediction of the distance of the responding animal and hence in the detection function. Since there is no other way to verify bearing error, I included the playback bearing from other observers to assess the bearing accuracy. I have found the average bearing error was around 10° (±10.85), leading to distance estimation of the sound source within an average range of 216.67 (±90.17) meters. The bearing error is less than in previous studies, where O'Gara et al. (2020) reported a 13.2° (±16.3) bearing error in a mock howling survey. They found the bearing error leads to animal position localisation within the radius of <1km from the source. The inclusion of the efficacy of acoustics triangulation is essential for density models. The error can be placed as a measurement error to estimate unbiased population density (Borchers et al., 2010).

I have identified 11000 km^2 wolf habitat in four districts of Maharashtra through a maxent distribution model using various presence locations. The low AUC value (0.54) is due to the smaller area. Although I had planned to conduct 100 howling surveys, I could not achieve the target due to logistical constraints and later due to Covid19 restrictions. However, my model performed well with an acceptable upper and lower limit due to a reasonable response rate in the howling survey. The estimation can be done more precisely through a landscape-level systematic grid howling survey.

The study aimed to standardise a population estimation method for wolves in a human-dominated landscape. Although the population distribution of wolves has been shrinking rapidly over the past century, their population status remained unassessed globally due to difficulties in censoring this visually cryptic species. Through the active acoustics survey, I found a population range of Indian wolves in the human-dominated landscape. I have also highlighted several prospects by which the model can give more precise and unbiased estimation through a larger sample size and identifying howls to an individual. The study will provide a guideline for wolf census for conservation biologists. The wolf population density as an apex predator determines habitat quality and sustainability. Therefore, the study is a stepping stone for using bioacoustics to estimate animal density and play a significant role in global wolf conservation.

5.5 Limitations of the current population estimation model and way forward

The study introduced three observer approaches to determine the distance of respondent wolves through an active howl survey. In the study, I used a single-directional microphone setup. But using three directional microphones, one with each observer, will allow combining the three recordings and obtain better howl quality which will be very useful in howl analysis. Also, if observer 1 fails to detect the response in a single microphone setup, the recording of the howl response will be missed entirely. I obtained howl responses in seven surveys, and the howl response survey points were very far from each other. Therefore, there were no chances of individual overlap in those responses, which made me assign each respondent animal a unique individual ID. In a larger sample size with close or repeated grid sampling, there might be a possibility of repeated capture of single individuals. Studies showed unknown individuals could be identified from their howls with 75% accuracy (Sadhukhan et al., 2021), but further identification accuracy is required for using them in the density model. Although scientists recently found that pack members shape the structure of chorus howl (Marti-Domken et al., 2022), and packs may have a signature howl (Zaccaroni et al., 2012), no method is available to identify groups from chorus howls. As the machine learning algorithm is refined every day, I can expect to develop a model to identify individuals or packs soon. But an extensive dataset of known howl samples is required to build such models. Identification of an individual or pack will enable using identity-based capture history through CMR. Combining the ASCR with identity-based CMR will reduce bias and heterogeneity in the model and provide robust estimation (Laake & Borchers, 2004; Marques et al., 2013). In identity-based CMR, individuals captured in one howling survey session can be recaptured in another session or year to provide additional information on survival, home range and pack dynamics.

CHAPTER 6 General discussion and potential research direction

6.1 Introduction

The Indian wolf is considered a Schedule I or endangered species in the Wildlife Protection Act 1972. Since they survive predominantly in a human-dominated landscape, they face immense survival threats due to habitat degradation and man-animal conflict (Agarwala et al., 2010; Habib & Kumar, 2007; Jethva & Jhala, 2004b). Besides these threats, their population status has remained unassessed over the years due to difficulties associated with the population estimation of this visually cryptic species (Cozzi et al., 2021). A few studies have suggested that around 1000 to 2000 (Sillero-Zubiri et al., 2004) wolves are left in India, but those are rough estimates without significant statistical evidence. Therefore, a non-invasive statistical tool is required to estimate this visually cryptic species. Since the howling survey is considered the most efficient monitoring tool for this visually cryptic species (Harrington & Mech, 1982), my study aimed to standardise a statistical tool to estimate the population of Indian wolves based on their howl. I have started my work with a single point of reference on Indian wolf vocalisation, where a comparative study was done to compare the Indian wolf howl with a few other subspecies (Hennelly et al., 2017). Therefore, I intended to characterise howl and other harmonic vocal calls to generate basic information about their howl, which had remained largely unexplored. Knowing their howling behaviour is the principal component in designing an efficient population estimation tool for the Indian wolf, which led me to study their vocal behaviour. In the population model, the identification of an individual always plays a crucial role as it allows Capture-Mark-Recapture (CMR) (Clutton-Brock & Sheldon, 2010), and it reduces bias and heterogeneity in the estimation (Marques et al., 2013). Therefore, identifying an individual based on their howl was one of the critical components of my study. Although my model showed a potential method to identify unknown howls, the model is yet to be improved with known training extensive data set to be compelling enough for the CMR model. Thus I have designed a study to estimate the population of Indian wolves using howl responses by the Acoustics Spatial Capture-Recapture model without the involvement of an identification based CMR model.

6.2 *Basic Characteristics of howl and other harmonics calls*

Broadly, the Indian wolf vocalises through two types of calls - harmonic calls (originated from the vocal fold) and noisy calls (originated from the resonating vocal tract) (Harrington & Mech, 1978b; Joslin, 1966). As harmonic calls are intended for long-distance communication, and my objective was to use long-distance communication for population estimation, I studied different harmonic calls. Harmonic calls are further subdivided into several subtypes, and each one is intended for a specific behaviour (Harrington & Mech, 1978b). In the first chapter, I classified different harmonic calls through unsupervised classification and found four types of calls. I described the behavioural significance of every vocal repertoire. Howl is very closely related to social squeak. The main difference lies in their duration and the coefficient of frequency variation, where howl is of a more extended (>5second) duration call than the latter. The frequency variation is high (coefficient of frequency variation = 18.778±3.587) in a social squeak. The segregation of different vocal types through the statistical model was crucial because aural estimation is often subjected to observer bias. Therefore, findings from this chapter are essential, and it generates the basic information on the vocalisation of the Indian wolf.

6.3 *Identification of howl to individual*

Studies over the years found that wolf howls contain individual-specific information (Fentress, 1967; Root-Gutteridge et al., 2014b, 2014a; Tooze et al., 1990). But identifying the unknown individual from their howls had remained challenging over the years, without which howl could not be used in the Capture-Mark-Recapture study (Marques et al., 2013; Stevenson et al., 2015). By understanding the importance of howl identification to an individual in population estimation, I trained a model using known howls and verified the model with a set of unknown howls (unknown to the model). In this supervised classification, I achieved 97.9% accuracy in identifying known howls (trained dataset) and 75% accuracy in identifying unknown howls (test dataset). For the first time, the unknown wolf howls were classified successfully. Although the achievement is very significant in wolf vocalisation research, further accuracy is required for using them in the population estimation model.

6.4 *Howling behaviour of Indian wolves and factors that influence them*

The howling behaviour of Indian wolves was never studied. Therefore, understanding the howling behaviour of the Indian wolf was the foremost concern before designing a howl survey methodology for population estimation. In the third chapter, I studied the howling behaviour of free-ranging wolves through the active howl survey response pattern. I highlighted the key behavioural aspect and the conservation importance of the Indian wolf. Based on the vocalisation behaviour, I found that a howl survey should be done during their pre-denning season (November-December). Additionally, wind speed is very low during this period. The best size for systematic grid howl sampling is 1.7×1.7 km^2. For an active howl survey, a 30watt speaker should be used with 3-5 trials. This chapter provided the crucial guideline for doing a howling survey in Indian conditions.

6.5 *Population estimation of a wolf using a howling survey*

In the final technical chapter of my doctoral thesis, I designed a howl survey based on the results from other chapters. In the newly designed triple observer survey, I obtained a relatively high howl response (seven out of twenty-five howl surveys) in randomly selected grids. I used 'redetection' at different points in space instead of using individual 'recapture' with time. Moreover, higher identification accuracy is recommended for using the CMR model in density estimation. Through my pilot study, I found that Indian wolf density is 3.65 individuals/100 km^2 with a lower limit of 1.67 to an upper limit of 5.63 (95% CI). Although I do not have data on the population density of Indian wolves to compare, the data and its error range are comparable with the population density of Iberian wolves, i.e. 2.55 wolves/100 km2 (95% CI = 1.87–3.51) estimated by DNA (scat) sampling by López-Bao et al. (2018). The standard error might decrease further with an increase in sampling effort for the population estimation of the wolf. This methodology can be a guideline for using the active howling survey in the population estimation of wolves globally.

6.6 *Research Achievements*

The study aimed to verify the feasibility of population estimation of the wolf through their long-ranging howl vocalisation. This study has successfully designed an unsupervised classification method to classify different call types. The methodology was adapted for categorising various calls of Dormouse (Marchewka & Postawa, 2019) and can also be useful for studying the vocalisation of other species. Through this study, I have classified various calls and described the significance of each vocal repertoire. I further explored supervised classification to identify individuals from unknown howls and achieved building a model to classify unknown howls for individual identification with 75% accuracy.

When I initiated my PhD, there was no information available on the howling behaviour of Indian wolves. Through my doctoral research, I was able to fill the research gap. The most significant accomplishment of the study is to standardise a cost-effective tool to estimate the population of Indian wolves through their long-range vocalisation.

6.7 Present Constrain and Future Directions

The population estimation of wolves is a global challenge. The methodology obtained from the study created a guideline and opened a new horizon in bioacoustics research. In Chapter 3, I developed a model through a new approach for identifying the individuals from their howl. Although the model showed a lot of potentialities, it is yet to achieve significant accuracy for using them in CMR based population model. Training the model with more howls and verifying them with a different test data set will increase its reliability. Continuous recording of captive individuals and recordings from free-ranging collared wolves for more extended periods will help obtain the required dataset. Radio collaring wolf with acoustics tag would be beneficial in generating the extensive data as are necessary for the howl identification model. Accelerometer data in the radio collar helps identify if the call originated from the focal animal (the collared individual). In my study, I used howl segments due to data limitations. As an alternative, using the whole howl series might improve the robustness of the model. Extracting howls from the chorus and identifying them to the individual is a challenge yet to be addressed as wolves mostly live in packs and predominantly howl together. The successful identification of howl to an individual will let us use individual capture history in the density model (Root-Gutteridge et al., 2014a; Stevenson et al., 2021). This will provide a ton of additional information, such as home range and survival rate.

The study introduced three observer approaches to determine the distance of respondent wolves through an active howl survey. In the study, I used a single-directional microphone setup. But using three directional microphones set up, one with each observer, will be very useful in howl analysis. Additionally, this will allow the recording of every howl response detected by any observers. Combining the ASCR with identity-based CMR will reduce bias and heterogeneity in the model and provide robust estimation (Laake & Borchers, 2004; Marques et al., 2013).

6.8 Concluding Remarks

This study showed the potentiality of the howl as a tool to identify individual wolves and the application of a howling survey for the population estimation of wolves. While the accuracy of identifying howl to an individual needs to be improved further to use them in CMR-based density estimation (individual recapture with time), ASCR (Redetection of howl response at a different point in space) provides an opportunity to estimate the population without identifying an individual. The methodology presented in the thesis was developed through a systematic study conducted in the human-dominated landscapes of Maharashtra. The fine-scale habitat utilisation data from radio telemetry and different presence data helped me delineate the wolf habitat precisely, which helped me choose the study area for the howling survey. The population status of the Indian wolf was unknown to date, but the methodology showed the potentiality of a way ahead. Our future endeavour with a systematic howling survey can be the front-runner towards successfully estimating the population of wolves in India.

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RESEARCH ARTICLE

Characterising the harmonic vocal repertoire of the Indian wolf (*Canis lupus pallipes*)

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Abstract

Vocal communication in social animals plays a crucial role in mate choice, maintaining social structure, and foraging strategy. The Indian grey wolf, among the least studied subspecies, is a social carnivore that lives in groups called packs and has many types of vocal communication. In this study, we characterise harmonic vocalisation types of the Indian wolf using howl survey responses and opportunistic recordings from captive and nine packs (each pack contains 2–9 individuals) of free-ranging Indian wolves. Using principal component analysis, hierarchical clustering, and discriminant function analysis, we found four distinct vocalisations using 270 recorded vocalisations (Average Silhouette width Si = 0.598) which include howls and howl-barks (N = 238), whimper (N = 2), social squeak (N = 28), and whine (N = 2). Although having a smaller body size compared to other wolf subspecies, Indian wolf howls have an average mean fundamental frequency of 422 Hz (±126), which is similar to other wolf subspecies. The whimper showed the highest frequency modulation (37.296 ±4.601) and the highest mean fundamental frequency (1708±524 Hz) compared to other call types. Less information is available on the third vocalisation type, i.e. 'Social squeak' or 'talking' (Mean fundamental frequency = 461±83 Hz), which is highly variable (coefficient of frequency variation = 18.778±3.587). Lastly, we identified the whine, which had a mean fundamental frequency of 906Hz (±242) and is similar to the Italian wolf (979±109 Hz). Our study's characterisation of the Indian wolf's harmonic vocal repertoire provides a first step in understanding the function and contextual use of vocalisations in this social mammal.

Introduction

Vocalisation plays a critical role in social animals for conveying information on foraging, reproductive, and social behaviours [1–7]. Characterising the vocal repertoire of a species provides a base for understanding the behavioural significance of different vocalisations and studying how vocal communication varies across populations, subspecies, and taxa [8–10].

The wolf (*Canis lupus*) is a social mammal and uses a variety of vocalisations for communication. Being present throughout Eurasia and North America, the wolf is one of the most widely distributed land mammals and occupies a wide range of different habitat types [11].

Competing interests: The authors have declared that no competing interests exist.

While there has been much research on wolves in North America and Europe, much less has been done on the wolves of Asia. For the grey wolf, most of the mitochondrial diversity is centred in southern and central Asia, where two independent and phylogenetically basal maternal lineages–the Tibetan and Indian wolf–are found [12–14]. The Tibetan and Indian wolf maternal lineages are estimated to have diverged around 700,000, and 300,000 years ago, respectively [12,15,16]. Despite its phylogenetic position as one of the oldest maternal lineages and among the smallest subspecies [12], relatively little is known about Indian wolf ecology and behaviour compared to other wolf subspecies. Studying the vocalisations of the Indian wolf can offer a greater understanding of the behavioural function of different vocal signals in Indian wolves and, more broadly, the variation in vocalisation and associated behaviour across subspecies and taxa within the *Canis* clade.

The best-known wolf vocalisation–the howl–is a long-range harmonic call used for territorial advertising and social cohesion [1,17–19]. Wolf howl acoustic structure has been shown to vary across individuals [1,20–24], groups [25], and subspecies [8,26]. Among the *Canis* clade, smaller species generally have howls that end in a sharp drop in frequency and a greater diversity of howl type usages [8]. Previous research has shown that Indian wolf howls generally have a higher mean fundamental frequency compared to other wolf subspecies, which may be attributed to its smaller body size [26]. Using a larger set of howls that are statistically classified by their acoustic features can provide a more robust description of the characteristics and diversity of Indian wolf howl types.

Along with the howl, wolves also communicate using seven to twelve other harmonic calls [27–29]. Harmonic calls are produced by the vibration of vocal folds in the larynx, which results in a series of multiple integral frequencies of the fundamental frequency [30]. Many of these other harmonic vocalisations are short-ranged, and due to difficulties in recording these calls, remain less studied compared to the wolf howl [31]. These short-ranged calls are essential for communicating passive or aggressive behaviour among social canids [31–33]. Grey wolves also use non-pitched or noisy calls, which are produced by the acoustic resonance of the vocal tract [19,34–36]. Instead of a specific frequency band, noisy calls possess concentrated acoustic energy around a particular frequency range. Therefore noisy calls do not have a clear pitch or distinct frequency band in their spectrograms [30].

The whimper, whine and yelp are various harmonic calls for communicating passive and friendly behaviour among wolves [19,32], whereas noisy calls such as growl and bark indicate varying levels of aggression [19,32]. The whimper, and whine vocalisations are similar to a crying sound with the whimper having a comparatively shorter duration than whine [19,34]. The whine vocalisation is mostly used for submissive behaviour, whereas the whimper is primarily used for greeting [19]. The yelp is a short and sharp cry vocalisation that is associated with submissive behaviour involving body contacts [19,34]. To communicate different levels of aggression behaviours, wolves use noisy calls, which consist of the growl, woof, and bark. Growl is a non-harmonic sound to show dominance in any interaction, whereas the woof vocalisation is a non-harmonic sound cue used by adults for their pups [19,34,37]. The bark is a short, low pitched sound with rapid frequency modulation and is used during aggressive defence [27,37], such as defending pups or defending a food resource [4,38]. Wolves also express communication through mixed vocalisation either by 'successive emission' or by 'superimposition' of two or more sound types [29]. A recent study on the Italian wolf (*Canis lupus italicus*) suggests six other types of calls may combine with howls to make a complex chorus vocalisation [39].

This study investigates the acoustic structure of harmonic vocalisations of Indian wolves and classifies these harmonic vocalisations using a statistical approach. We accumulated the vocalisation data from free-ranging and captive Indian wolves, which will be the first study to evaluate different types of vocalisations of this wolf subspecies. Using multivariate analyses, we describe and classify different harmonic calls to develop a vocal repertoire of the Indian wolf.

Materials and methods

Study species

The Indian wolf (*Canis lupus pallipes*) is among the smallest wolf subspecies with an average body weight of 20.75 kg [40]. Indian wolves are mostly found in grasslands and the edges of dense tropical deciduous forest on the Indian subcontinent [40–44]. The average home range of a pack varies from 180–250 km^2 [40]. We recorded vocalisations from nine packs of free-ranging wolves and ten captive wolves from Jaipur Zoo. For captive wolves, we collected vocalisation data from 10 wolves: two adult pairs and six subadults. One adult male was recently captured from the wild near the city of Jaipur, Rajasthan, India. The rest of the Indian wolves are descendants of captive breeders at Jaipur Zoo.

Study sites

This study was conducted in the state of Maharashtra (Fig 1) and Jaipur Zoo of Jaipur, Rajasthan, India. The study site in Maharashtra was located on the central Deccan Plateau [45], which consists of the overlapping habitat of tropical dry deciduous forest, grassland, savanna (Western part) and tropical moist deciduous forest (Eastern part) [46].

Data collection

Vocalisations of free-ranging wolves were recorded through acoustics survey from November 2015 to June 2016. The majority of the long-distance vocalisation recordings were collected through howling surveys to elicit howl behaviour. Opportunistically, spontaneous howls were also recorded. For other types of vocalisation data, we relied on opportunistic recordings from free-ranging wolves and captive wolves. Howl surveys were performed during early morning and evening hours using pre-recorded howls that were previously recorded from the Jaipur Zoo Indian wolves. Each howling session consisted of five trials with three minute long intervals [3]. A 50-second-long pre-recorded sequence of a solo howl was played three times using JBL Xtreme speakers (Harman Internation Industries, 2014) in order of increasing volume [3]. The session was followed by two 50-second-long chorus howls. In the case of a howling response, the session was terminated and repeated after 15 to 20 minutes [3]. Responses were recorded using Blue Yeti Pro Microphone (Blue Microphone, 2011) attached with Zoom H4N Handheld Audio Recorder (Zoom Corporation, 2009) at a sampling rate of 44.1KHz on 16-bit depth with 80 Hz noise filter. Along with howl surveys in the field, opportunistic recording sessions were conducted near wild Indian wolf den sites and rendezvous sites. In addition to howl surveys at Jaipur Zoo, vocalisations of captive wolves were recorded by installing microphones in the front of cages during closing hours (6:30 pm-7: 30 am).

Ethical approval

The study on captive wolves in zoos was done with the permission of the Director of Jaipur Zoo and the Forest Department of Rajasthan, India [Letter no- 3(04)-II/CCFWL/2013/4586-87; Dated 30th Oct 2015]. The survey of free-ranging wolves of Maharashtra was performed with the consent of the Principal Chief Conservator of Maharashtra Forest Department [Letter no- 22(8)/WL/CR-947(14–15)/1052/2015-16; Dated- 6th Aug 2015]. No animal was harmed during the study, and the standard non-invasive protocol of howling survey was maintained.

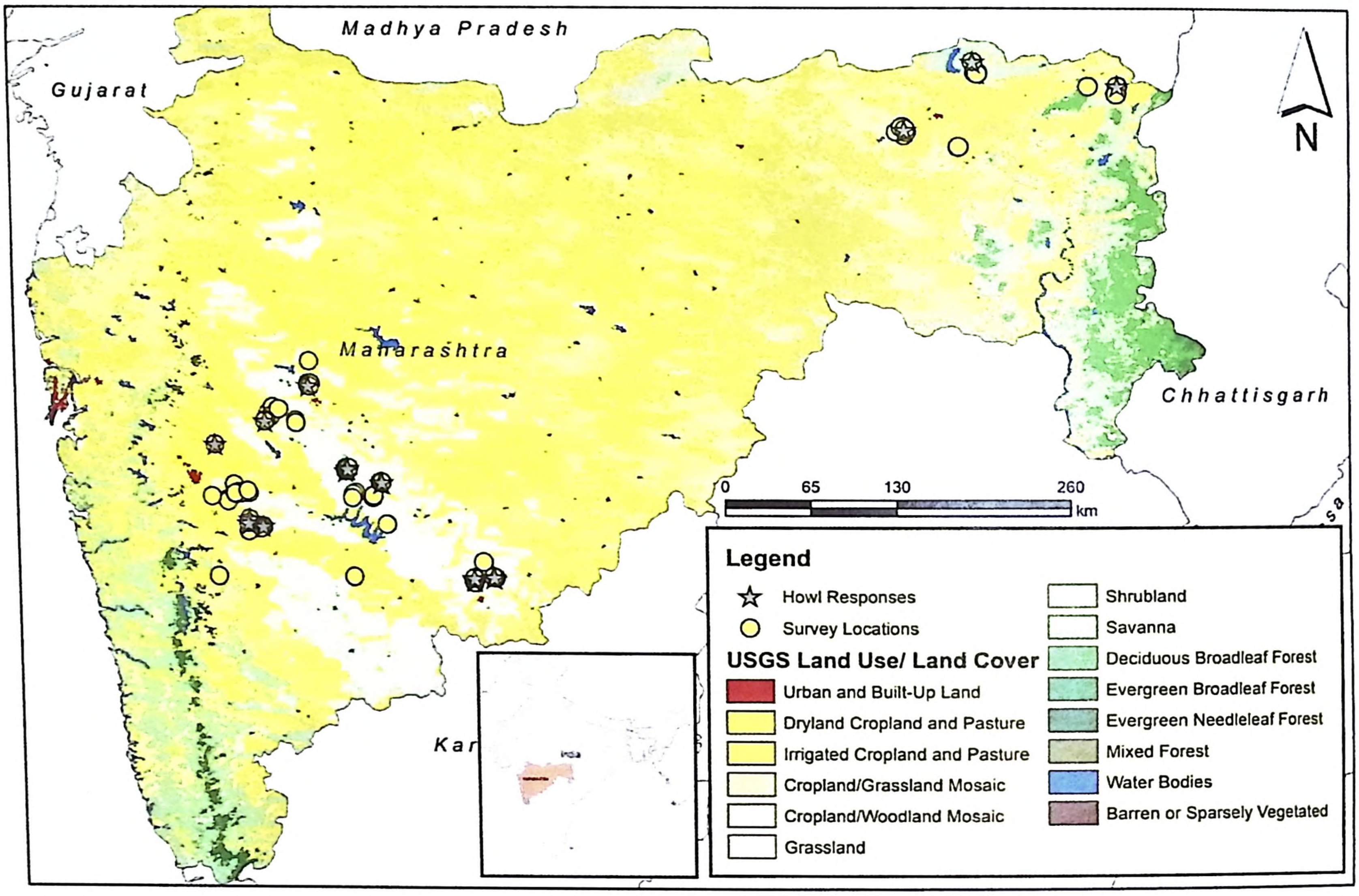

Fig 1. Map of survey sites of the free-ranging wolves. (Spatial Data Source: Political boundaries from Natural Earth, and Land Use/Land Cover data from USGS Earth Resources Observatory and Science (EROS) Center).

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Feature extraction

We focused our analysis on harmonic vocalisations and excluded noisy calls since they do not possess a clear spectral band. Spectrograms of each vocalisation were generated through the Raven Pro 1.5 software [47] using the *Discrete Fourier Transform* (DFT) algorithm. The discrete Fourier function transforms the same length sequence of equally spaced sample points (N, where N is a prime number) with circular convolution being implemented on the points [48]. *Hann windows* were used at the rate of 1800 samples on 35.2 Hz 3dB filter. A total of 270 spectrograms were selected for further analysis based on clarity (i.e. clearer spectrogram with low noise and without external sound overlap). Web plot digitiser v3 [49] was used for digitising fundamental frequency from the spectral images. This digitised data was obtained at 0.1sec resolution. From this data, eleven acoustic variables (Table 1) were obtained based on their performance from previous studies [20,22].

Statistical analysis

Principal Component Analysis (PCA). To obtain a smaller set of variables that explain most of the dataset's variation, we used a principal component analysis (PCA), which is an unsupervised statistical approach that extracts linearly uncorrelated variables from a suite of potentially correlated variables [50]. To simplify the interpretation of factors, we performed

Table 1. Acoustic variables based on fundamental frequency (f_0) that were extracted for this study.

Variable Name	Definition of Variable
Min f	The minimum frequency of the fundamental (f_0)
Max f	The maximum frequency of f_0
Range f	Range of f_0; f_0 = Max f–Min f
Mean f	Mean frequency of f_0 at 0.1 s interval over the duration
Duration	Duration of Howl measured at f_0; Duration = t_{end} - t_{start}
$Abrupt_{0.025}$	Number of abrupt changes in f_0 more than 25Hz at single time step (0.1sec)
$Abrupt_{0.05}$	Number of abrupt changes in f_0 more than 50Hz at single time step (0.1sec)
$Abrupt_{0.1}$	Number of abrupt changes in f_0 more than 100Hz at single time step (0.1sec)
Stdv	Standard Deviation of f_0.
Co-fm	Coefficient of frequency modulation of $f_0 = \Sigma\|f(t)-f(t+1)\|/(n-1) \times 100/\text{Mean } f_0$
Co-fv	Coefficient of frequency variation of f_0 = (SD/mean) X 100

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varimax rotation using Kaiser normalisation [51]. From our dataset of 270 vocalisations, we used eight scalar variables that are related to spectral structure (Range f, Duration, $Abrupt_{0.025}$, $Abrupt_{0.05}$, $Abrupt_{0.1}$, Stdv, Co-fm, Co-fv) for PCA analysis through the software SPSS (v22). The first principal component (PC1) and second principal component (PC2) were used in the subsequent clustering analyses.

Cluster analysis. To classify the recorded vocalisations from the Indian wolf, we used agglomerative hierarchical clustering through the R package AGglomerative NESting (AGNES)[52]. The agglomerative hierarchical clustering algorithm measures the dissimilarity between single and groups of observations using a "bottom-up" approach, thereby constructing clusters [53]. Agglomerative hierarchical clustering was performed using Euclidean distances with PC1 and PC2 from the 270-vocalisation data using eight scalar variables. Subsequently, *silhouette clustering* was combined with AGNES to validate the number of clusters in our vocalisation data. Silhouette clustering measures the similarity of observation within its cluster compared to other clusters [54]. The average silhouette value (0 represents poor fit, 1 depicts the highest fit) describes *the evaluation of clustering validity* [54]. Average Silhouette width (S_i) was calculated for 14 different solutions (2 to 15 clusters). The "solution" that provided the best fit was selected upon the maximum average silhouette value. The dendrogram was plotted using '*Circlize Dendrogram*' in the package '*Dendextend*' in program R [55].

Discriminant Function Analysis (DFA). Discriminant function analysis (DFA) was performed using PC1 and PC2 as an independent variable under the program SPSS (v22) to cross-validate the obtained clusters from AGNES. Predicted clusters that were determined by the maximum silhouette value were then used as a grouping variable to evaluate within-group covariance in DFA analysis. From these clusters, we then used the box plot to show the overall pattern and distribution characteristics of different vocal clusters.

Results

Principal component analysis

Two principal components (PC1 and PC2) were generated from the eight simple scalar variables through PCA based on Kaiser-Guttman Rule (Eigenvalue >1) [56]. PC1 and PC2 together explained 70.6% variance. PC1 was based on the variances of six acoustic parameters ($Abrupt_{0.025}$, $Abrupt_{0.1}$, $Abrupt_{0.05}$, Co-fv, Range f, Stdv) whereas PC2 is explained by the variances of five parameters ($Abrupt_{0.1}$, Co-fm, Duration, Range f, Stdv) (Table 2).

Table 2. **The loadings of PC1 and PC2.** Communalities are the proportional factors by which the importance of each variable is explained.

Acoustics Parameters	PC1 Loading after varimax rotation	PC2 loading after varimax Rotation	Communalities
$Abrupt_{0.025}$	.858	0	0.741
$Abrupt_{0.1}$	.602	.415	0.535
$Abrupt_{0.05}$	.825	0	0.732
Co-fm	0	.945	0.898
Co-fv	.862	0	0.750
Duration	0	-.382	0.231
Range f	.852	.392	0.880
Stdv	.759	.556	0.885
% of Variance	48.946	21.692	

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Cluster analysis

The highest silhouette value ($S_i = 0.598$) was obtained at the 4-group solution in the cluster analysis using PC1 and PC2 from PCA analysis (Fig 2). The average silhouette value was 0.62 for the first cluster (N = 238), 0.37 for the second cluster (N = 2), 0.38 for the third cluster (N = 28) and 0.73 for the fourth cluster (N = 2) (Fig 3). The 4 clusters were formed at 3.9 clustering scale through agglomerative hierarchical clustering (Fig 4)

Discriminant Function Analysis (DFA)

DFA achieved 95.9% accuracy of vocal group identification using two PCA values (Table 3). Each of the four groups has a distinct group centroid. The graphical representation using two discriminant functions (DF1 and DF2) shows that vocal clusters do not overlap (Fig 5).

The whisker box plot represents the variation among acoustic variables within the four identified call types (Fig 6). Call type 1 had the longest duration (5.214±2.49 Sec) whereas call type 2 showed the shortest duration among the four recognised groups (0.4±0) (N = 2). Type 2 calls also have high-frequency modulation (37.296±4.601) (variation in frequency per unit time). However, frequency variation (around the mean) is highest in type 3 calls (18.778 ±3.587) (Table 4).

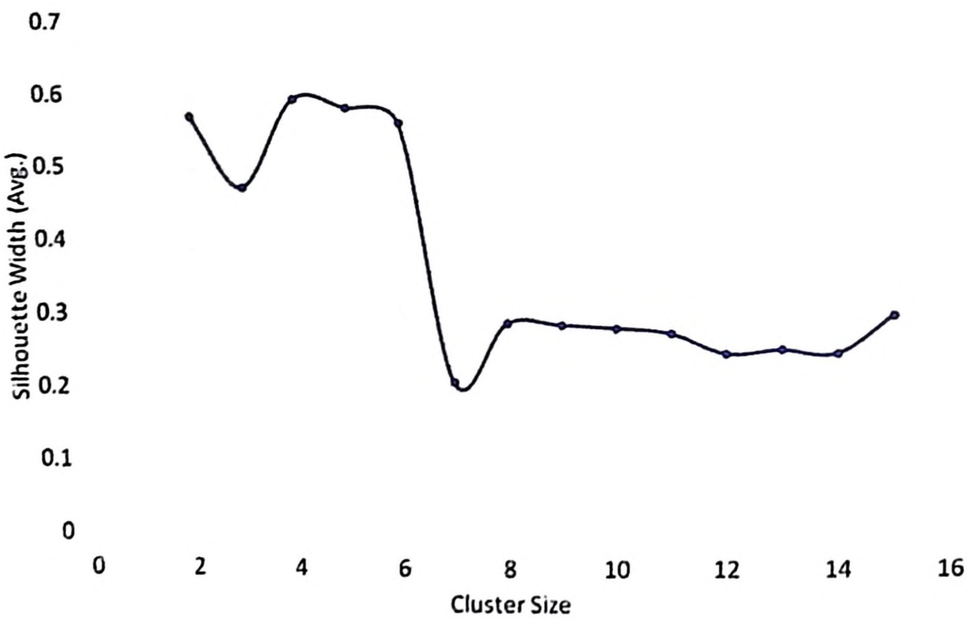

Fig 2. Average silhouette width plotted against 14 different solutions (2–15 cluster). Average Silhouette width represents the significance level (0 represents poor fit, 1 represents best fit). We obtained maximum silhouette width in 4 cluster solutions, i.e. $S_i = 0.598$.

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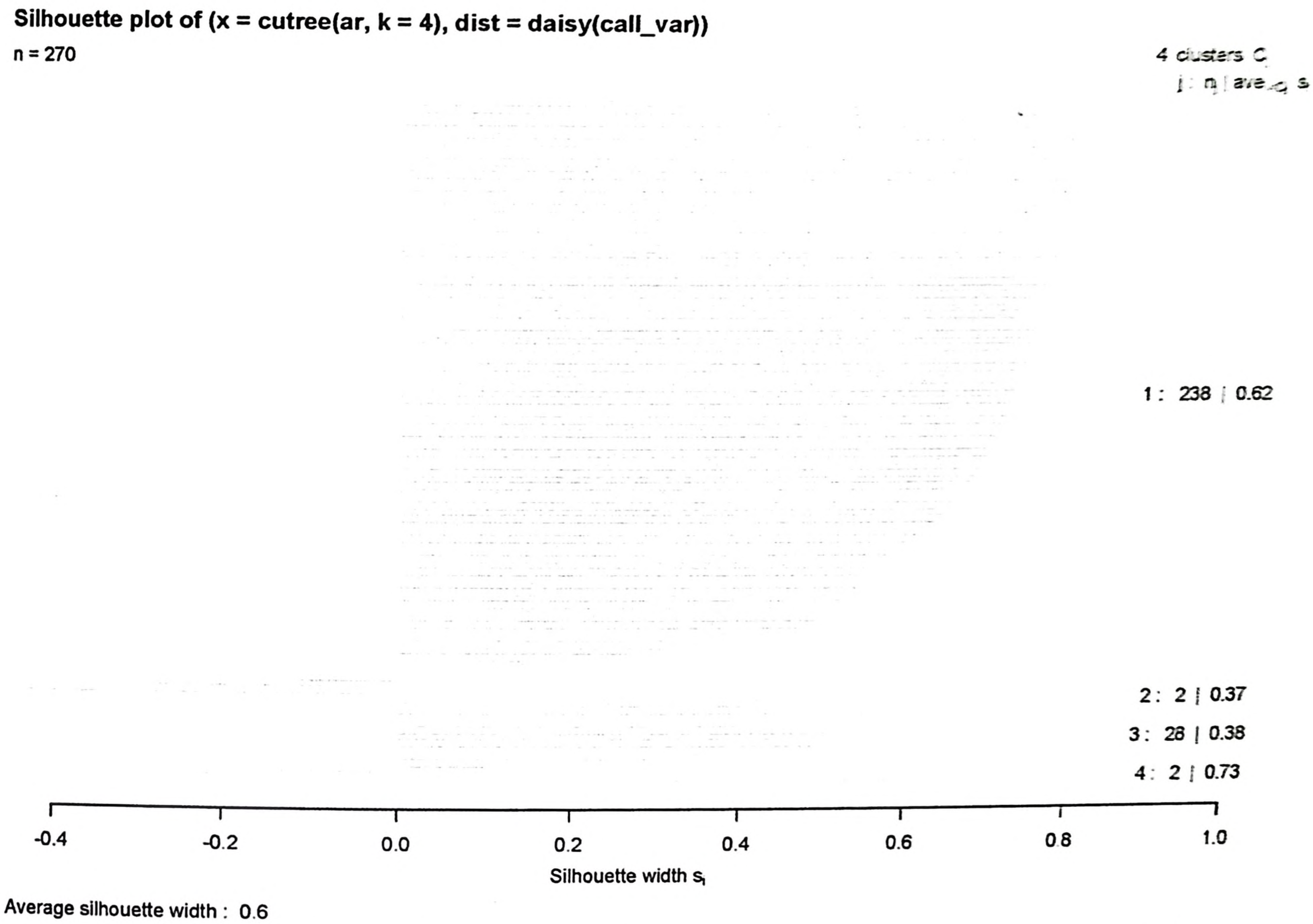

Fig 3. Silhouette plot showing the validation for the consistency among the clusters. This plot assesses the similarity or difference of each call from its clusters. A negative value indicates the chance of a call to fall under the neighbouring cluster. The average silhouette value of 4 groups are 0.62 (N = 238), 0.37 (N = 2), 0.38 (N = 28), 0.73 (N = 2) respectively.

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Discussion

This study provides a quantitative assessment of the vocalisations of the Indian wolf subspecies. Our results show that there are four statistically classified groups of Indian wolf vocalisations based on ten captive individuals and nine free-ranging Indian wolf packs. Though the Four to Six solution groups showed a narrow difference in their average silhouette values based on *silhouette plot* analysis, the four cluster solution was found to be the most significant based on the global maxima. This characterisation of vocalisations provides a first step to evaluating the function and contextual use of different types of vocalisations in these canids.

The first, most prolonged (5.214±2.49 sec) call type in our dataset is identified as a howl (Fig 7a). The fundamental frequency of the howl ranged from 359 Hz (±116) minimum to 469 Hz (±141) maximum (N = 238). Despite having a smaller body size, the mean fundamental frequency of the Indian wolf howl (422±126 Hz) was similar to the mean fundamental frequency of other wolf subspecies reported in previous studies [26]. This contrasts previous research that described Indian wolves as having a higher mean frequency in howls [26]. Our study's large sample size of individuals and use of a classification model to statistically discriminate

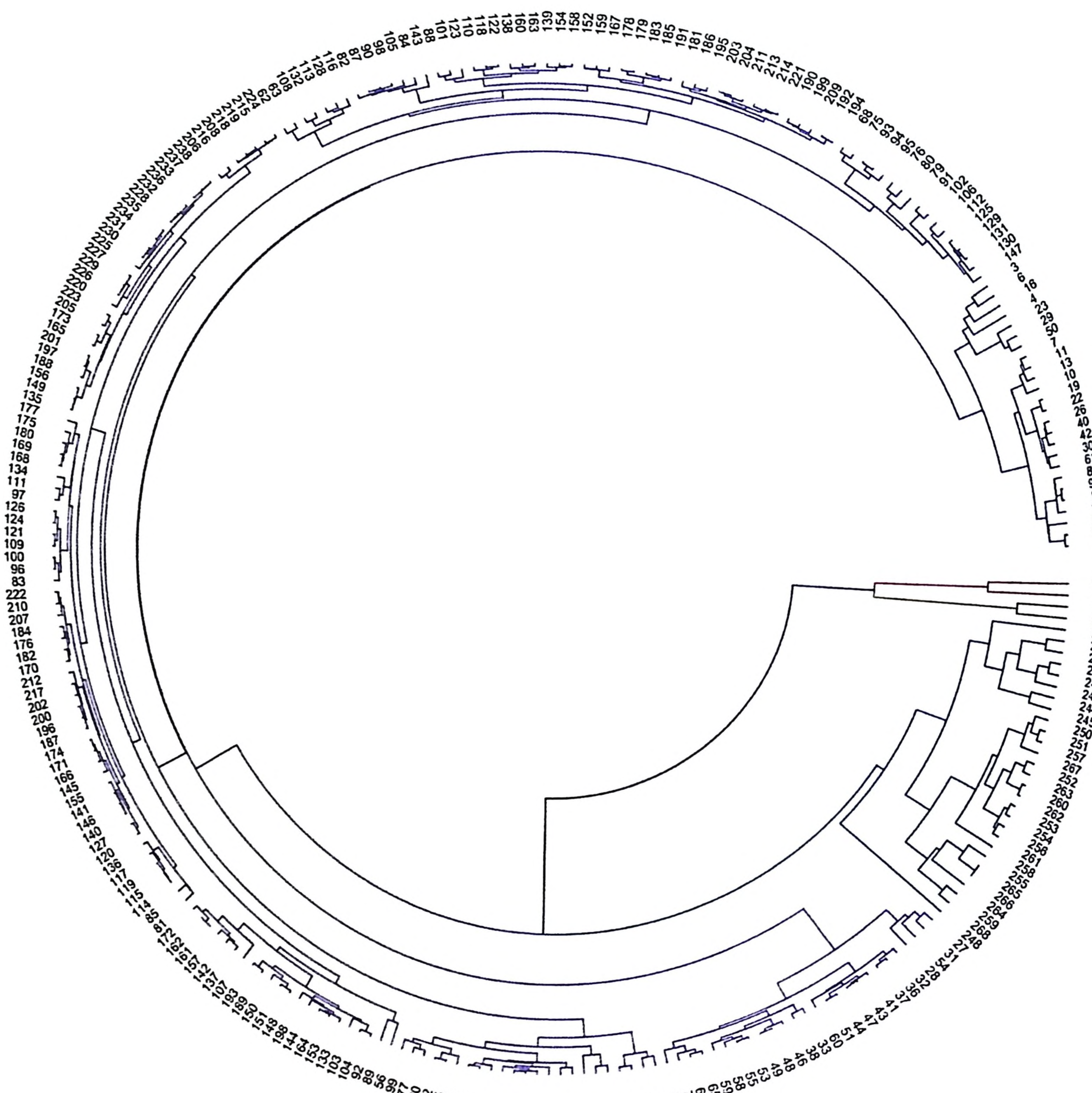

Fig 4. Cluster Diagram obtained from Agglomerative hierarchical clustering using Euclidean Distance as matric function. Four clusters were formed at 3.9 Clustering scale. Cluster 1 (Howl), Cluster 2 (Whimper), Cluster 3 (Social Squeak) and Cluster 4 (Whine) are denoted by the colours blue, red, green and brown, respectively.

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vocalizations may have aided in excluding other vocal types–such as howl barks–in our analyses to robustly describe Indian wolf howls. Additionally, variation in howl acoustic structure has been suggested to be partly individual-specific, which may be due to a combination of differences in body sizes, age class, or gross anatomy [1,20–24]. For example, the mean fundamental frequency of 11 Iberian wolf individuals was reported to range from 332Hz (±47) to

Table 3. Classification results of Discriminant Function Analysis (DFA). 95.9% of the vocal clusters (estimated from Agglomerative hierarchical clustering) are identified correctly.

		Cluster	Predicted Group Membership				Total
			1	2	3	4	
Predicted in Cluster analysis	Count	1	229	0	8	1	238
		2	0	2	0	0	2
		3	0	0	26	2	28
		4	0	0	0	2	2
	% Correct	1	96.2	.0	3.4	.4	100.0
		2	.0	100.0	.0	.0	100.0
		3	.0	.0	92.9	7.1	100.0
		4	.0	.0	.0	100.0	100.0

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666Hz (±60) [21]. This high acoustic variation associated with individual wolves highlights the importance of having a large enough sample size of individual wolves to robustly describe vocal types and assess individual-specific variation within a population. To further understand the influence of body size on wolf howl acoustic structure, it would be important to identify howls using a classification-guided approach across all vocalization data of various subspecies as well as incorporating information of each howl's associated wolf weight and individual's identity.

Since the howl is the most detectable vocalisation used in long-range social cohesion and territorial advertisement [8,31], our high howl sample size shouldn't be considered as the most frequent vocalisation. Barking-howl, which was mentioned by many authors as a common type of mix vocalisation in wolves [31,35,39], falls under the same cluster along with howling (Fig 7b). From our field observations, wolves bark in defence to an immediate threat. In one

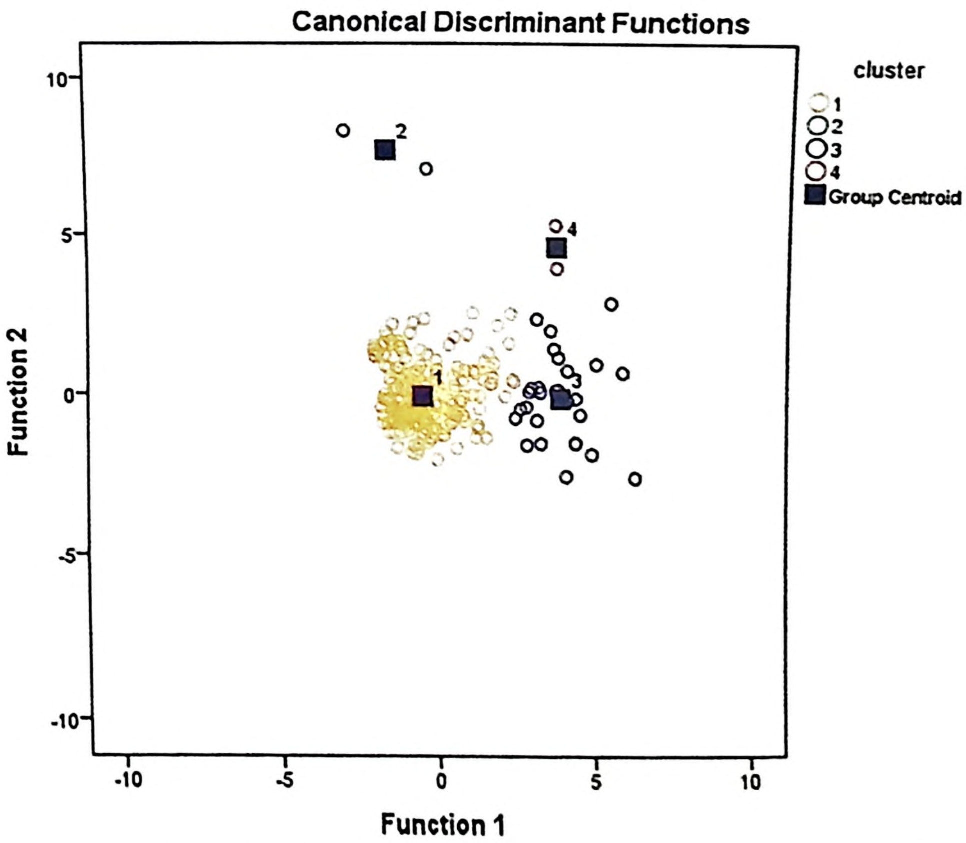

Fig 5. Plot for Discriminant Function Analysis (DFA) using PCA values for 270 vocalisation data from the Indian wolf. Different colours represent different call type.

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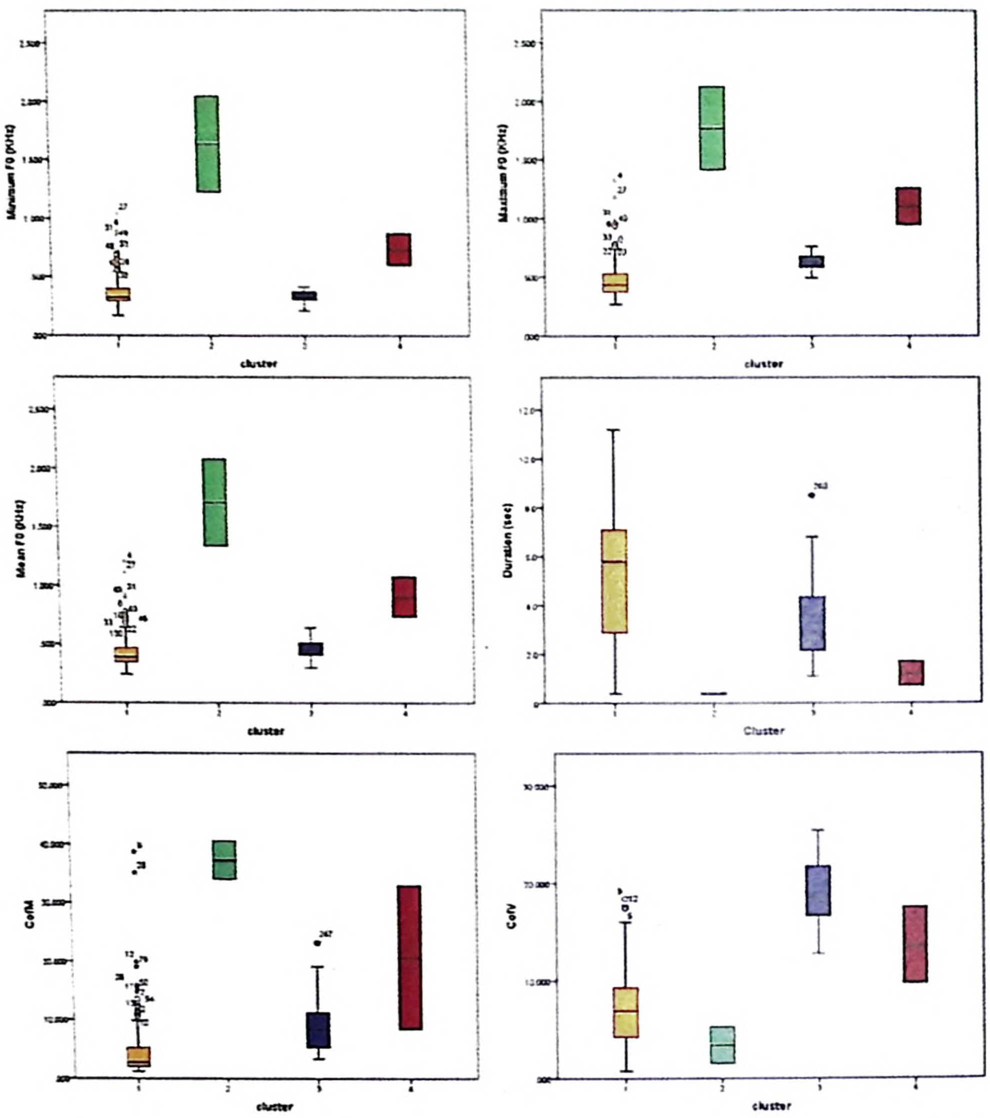

Fig 6. Box plot of variation among acoustic variables between different call type. a. Variation among minimum frequency, b. Variation among maximum frequency, c. Variation among Mean frequency, d. Variation among duration of the call, e. Variation among coefficient of frequency modulation, f. variation among coefficient of frequency variation.

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such occasion, the she-wolf of a pack started barking at nearby villagers to protect her pups and did not stop until all the three pups ran away to a safer distance from the villagers.

While the howl has been extensively studied in its behavioural function and variation across subspecies [8,26], less is known about short-range communication among wolves. Our study has identified and described three short-range communication call-types found in Indian wolves. Corresponding to our results, the second call type has the highest frequency modulation (37.296±4.601) and is commonly known as a whimper (Fig 7C). The whimper is low intensity but high-pitched sound that is used for short-distance communication among pack

Table 4. Variation among important acoustic variables within the four identified call types.

Cluster	Min f_0 (Hz)	Max f_0 (Hz)	Mean f_0 (Hz)	Range f_0 (Hz)	Duration (Sec)	Co-fv	Co-fm
1	359±116	469±141	422±126	110±65	5.21±2.49	7.17±3.689	4.444±4.463
2	1632±578	177±5	1708±524	137±77	0.4±0	3.407±2.632	37.296±4.601
3	327±51	623±77	461±83	295±50	3.47±1.85	18.778±3.587	9.071±4.802
4	733±190	1100±220	906±242	367±29	1.2±0.70	13.649±5.526	20.694±17.347

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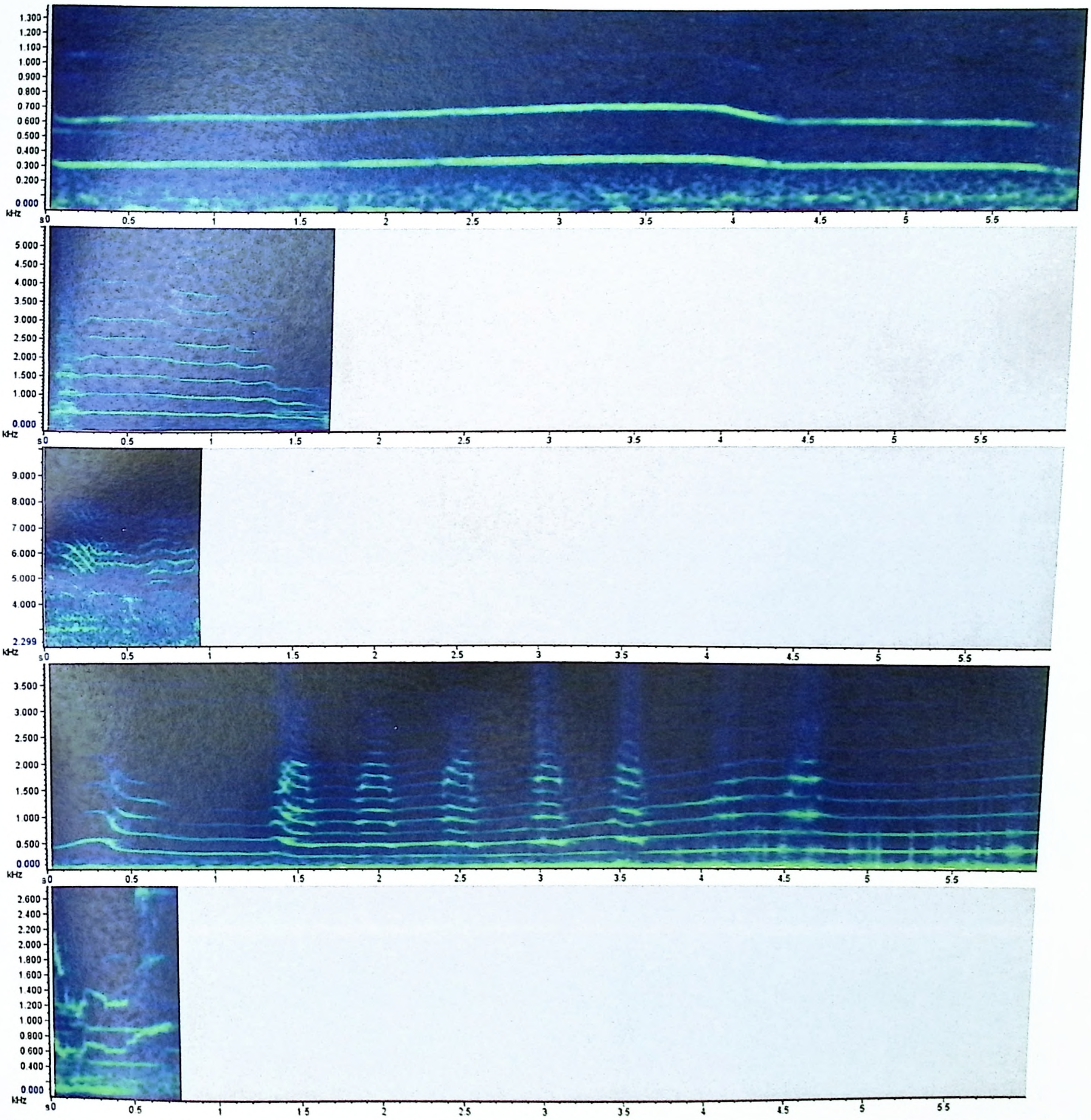

Fig 7. Spectrograms of different types of vocalisations of the Indian wolf. a. Howl (type 1); b. Bark-Howl (type 1 subtype); c. Whimper (type 2), d. Social Squeak (type 3); e. Whine (Type 4).

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members [28,31] (mean fundamental frequency = 1708±524 Hz). This short duration (0.4±0 sec) vocalisation is reported to be associated with submissive or friendly greeting behaviour [31,35]. Since it is not audible from more than one to two hundred yards away [35], our dataset contains only a few observations (from different packs) of this type of call (N = 2). While our study provides some initial insight into the acoustic structure of this vocalisation, further sampling will be needed to characterize the acoustic structure of the whimper robustly.

The third group of Indian wolf vocalisations can be termed as *'social squeak'* (Fig 7C), following observations by previous studies (Mech 1981, Crisler 1959, Fentress 1967). This high-frequency variable vocalisation (18.778±3.587) in the Indian wolf is similar to 'talking', which was defined as 'hovering around one pitch' [57]. The social squeak is considered to be context-dependent, with variation within the call type being dependent on differing social interactions among individuals [58]. Otherwise, there is little known about its function in wolf packs and if it's a common communication across different canid species and within domestic dogs. Our results suggest that the social squeak has a minimum frequency of 327 Hz (±51) to Maximum of 623 Hz (±77) for Indian wolves (N = 28).

Lastly, our fourth vocal group we identified as the whine (Fig 7E), which is characterized as a short duration vocalisation (1.2±0.707 Sec). The whine is mainly used during stressful situations, such as pack separation and/or intra-pack conflict [30]. Additionally, female wolves have also been reported to whine during the nursing of pups in the den [59]. The whine in the Indian wolf (*Canis lupus pallipes*) is longer than the whine reported in Italian wolf (0.13±0.10 sec), which the Indian wolf has comparatively larger body size than Indian wolf [39]. Although our data set is too small (N = 2; from two different individuals) to interpret robustly, the mean fundamental frequency of Indian wolf whine (906±242 Hz) has a similar frequency as the Italian wolf (979±109 Hz) [39].

Arising from the challenges of monitoring elusive and low-density species, acoustic methods for detection and estimating population parameters has become increasingly utilized in wildlife management [60,61]. Early wolf biologists had recognized its effectiveness for detection [62], and further statistical work on howl acoustic structure has improved its ability to monitor wolf populations [63–65]. Statistically validating wolf howls from other vocalisations using an unsupervised classification technique avoids having a human biased sample of vocalisations for performing subsequent behavioural and statistical analyses, such as for identifying individuals [43]. It is important to note that howls can be context-dependent, in which individuals' howl acoustic structure can vary according to certain behavioural contexts [24]. Since the howls were recorded from both elicited and spontaneous responses, our study's characterization of the howl should be taken with caution, as it may comprise of multiple context-specific howls.

Further research on a larger dataset of Indian wolf vocalisations can develop a more robust classification of the vocal repertoire of this subspecies. Additionally, we defined call types in our study based on similarity to previously defined call types, such as whimper, whine, and social squeaks [35,66]. Incorporating information on the behaviour associated with these call types would aid in describing and validating the call types in our study. Therefore, statistical classification coupled with behavioural monitoring through a visual recorder is one future avenue of research, which will aid in decoding wolf behaviour in the context of its vocalisation. More broadly, the species within the *Canis* clade vary in their body sizes, social structure, and habitats [67]. The diversity of social complexity and vocal communication across species within *Canis* represents a unique system to address questions on the relationship between vocal communication and social complexity [68–70]. Therefore, describing the vocal repertoires of various canid taxa provides a first step into understanding the ecological, social, and phylogenetic factors influencing the diversity of vocal communication within the genus *Canis*.

Supporting information

S1 File. The variables, clusters and other details information of every calls.
(PDF)

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Identifying unknown Indian wolves by their distinctive howls: its potential as a non-invasive survey method

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Previous studies have posited the use of acoustics-based surveys to monitor population size and estimate their density. However, decreasing the bias in population estimations, such as by using Capture–Mark–Recapture, requires the identification of individuals using supervised classification methods, especially for sparsely populated species like the wolf which may otherwise be counted repeatedly. The cryptic behaviour of Indian wolf (*Canis lupus pallipes*) poses serious challenges to survey efforts, and thus, there is no reliable estimate of their population despite a prominent role in the ecosystem. Like other wolves, Indian wolves produce howls that can be detected over distances of more than 6 km, making them ideal candidates for acoustic surveys. Here, we explore the use of a supervised classifier to identify unknown individuals. We trained a supervised Agglomerative Nesting hierarchical clustering (AGNES) model using 49 howls from five Indian wolves and achieved 98% individual identification accuracy. We tested our model's predictive power using 20 novel howls from a further four individuals (test dataset) and resulted in 75% accuracy in classifying howls to individuals. The model can reduce bias in population estimations using Capture-Mark-Recapture and track individual wolves non-invasively by their howls. This has potential for studies of wolves' territory use, pack composition, and reproductive behaviour. Our method can potentially be adapted for other species with individually distinctive vocalisations, representing an advanced tool for individual-level monitoring.

Accurate population estimates are a critical part of wildlife biology, conservation and inform management strategies[1]. Informed management decisions rely on accurate estimates which can be hard to achieve but are critical as the conservation status of any species is dependent on its population size, which is inversely correlated with extinction risk[2]. Therefore, it is of the foremost importance to have a statistically robust population estimation technique. However, widely used population estimation methods such as camera trapping and sighting-based distance sampling fall short in analysing population trends of certain elusive species or species living in extensive home ranges[3,4]. Many of these species are vocally active, which inspired scientists to study the effectiveness of an acoustics-based population abundance model for these species[5–8]. Acoustic monitoring has long been used to monitor the presence of aquatic animals, amphibians, insects, and birds[9–13]. The critical advantages of acoustic monitoring are that it can be operative at day and night[11] and detect visually cryptic species or those spread over large home ranges[7,15,16]. Like camera traps, passive acoustics devices can operate throughout the day for weeks without intervention, and this perpetual data can be analysed easily with the advancement of methodologies for automating the process[17]. Recordings from these devices can be used in calculating a wide range of metrics including acoustic indices[18,19], animal diversity[19,20], animal localisation[21–23], and density[24,25] estimation. This density estimation is mostly obtained through Spatially Explicit Capture-Recapture (SECR) that relies on multiple recording stations for Capture-Mark-Recapture (CMR), and instead of 'recapture' with time, it considers 'redetection' in different points in space[24–26]. This methodology is applied to individuals that are not identifiable from their calls[25,27]. The conventional CMR model requires identification at the individual level[27,28], but it provides a robust population estimation[28] and much additional information such as home-range, survival rate, animal movement pattern, and population viability analysis[29]. However, the difficulty of successfully identifying unknown individuals from their calls has prevented its use for more species, though new techniques are being

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developed for some species, including the use of unsupervised classifiers to cluster calls[30]. Here, we explore the potential of identifying individuals through supervised classification from their vocal features to potentially improve identification to the point where CMR surveys are possible for an elusive and wide-ranging species.

Like other grey wolf subspecies, Indian grey wolves are known for their long-ranging communication via howls[31]. Howling is a social communication process, vital for the overall behaviour of many canid species[32]. It has evolved in wolves to communicate with other group members over a long distance as well as to demarcate their vast territories[33]. Due to its high amplitude and low frequency, a howl can travel for six kilometres or more[34–36]. Hence, an acoustics survey using howling may be more advantageous than a visual survey or other methods, such as snow-tracking[22,23,35,37]. As vocalisations of wolves were found to be highly variable within and among individuals[31,38], the howl is a useful tool to identify individuals[39–41]; thus, wolves are ideal candidates for acoustic monitoring techniques.

Previously the '*Howlbox*', a self-contained active acoustics-monitoring device that broadcasts howls and records the responses automatically, was tested for its capability to detect wolves[42,43]. This device was unsuccessful in surveying wolves due to low detection rate as, instead of howling back, the wolves visited the device site without howling, and various technical failures[42]. A few studies using passive acoustic devices show the potentiality of successful localisation and monitoring of the grey wolf[23,44]. However, these only allowed for presence to be detected and stopped short of individual identification. In contrast, the identification of wolves from their distinctive howls will open an opportunity for more conventional CMR methods[45], and this will improve population estimation without bias and help to measure other ecological variables, such as site occupancy and home-range. With the ability to identify individual wolves from howl recordings, information on population sizes, dispersal patterns, pack composition and the presence of pups could be obtained. These would be used to develop conservation management strategies and to examine population trends with howl surveys conducted over multiple years. Therefore, our study aimed to record howls from Indian wolves (*Canis lupus pallipes*) and test the feasibility of identifying unknown individuals from their howls alone using a supervised classification method.

Methods

Study species. Indian wolf, subspecies of the grey wolf is among the keystone species found in the Central Indian landscape[46] and reside in arid grasslands, floodplains, and the buffer of dense forests[16–19]. The Indian wolf plays a significant ecological role in controlling ungulate populations in the human-dominated landscapes[50,51]. The population status of Indian wolves is entirely unknown[52]. It is known that Indian wolves face a major threat from humans as their habitat is increasingly used by humans, and human-wildlife conflict is increasing[53]. Therefore, time is a critical factor to their conservation. The major challenges for population estimation of the wolf are its vast home range of ~ 230 km^2[48] and that they actively avoid camera traps because of camera sound, light, and odour emission[54]. Since implementing standard population monitoring tools in these landscapes is a tremendous challenge, monitoring their population through howls can be an essential technique. The average fundamental frequency and duration of Indian wolf howls are 422 Hz and 5.21 s, respectively[55]. Due to its low-frequency range and longer duration, it can be heard from an extended distance like howls of other subspecies[23,35,36].

Study site. The study was conducted on captive individuals of Jaipur Zoo and free-ranging, wild wolves of Maharashtra, India.

Jaipur Zoo is situated at the heart of Jaipur City, Rajasthan, India. Since Jaipur is one of the major tourist destination and capital of Rajasthan, the anthropogenic noise is reasonably high in and around the zoo. All the wolves (n = 10) in Jaipur zoo were offspring of captive-bred individuals except one adult male recently captured from a wild population of Rajasthan.

The data of free-ranging wild wolves were collected from six districts of Maharashtra. Pune, Ahmednagar, Solapur and Osmanabad (Fig. 1) districts fall under the semi-arid drought-prone area of the Deccan peninsula Biogeographic Zone (Zone 6)[56]. The dominant habitat type in our sampling areas was *Deccan thorn scrub forests*[57]. The terrain is gently undulating with mild slopes and flat-topped hillocks with intermittent shallow valleys, which forms the primary drainage channels. Grassland area is distributed in fragmented patches, creating a mosaic of grazing land, agricultural land and human settlements. Striped hyenas (*Hyaena hyaena*), golden jackals (*Canis aureus indicus*), and Indian leopards (*Panthera pardus fusca*) are the co-predators in this landscape[18,58]. Wild prey include blackbucks (*Antilope cervicapra*), chinkaras (*Gazella bennettii*) and wild pigs (*Sus scrofa cristatus*); but a significant part of their diet is domestic livestock[18,50,59].

In Maharashtra, Nagpur and Gondia districts come under the central Deccan Plateau with Tropical dry deciduous broadleaf forests[56,57]. Due to moderate to high rainfall, vegetation is dense in most of the areas. Our sampling areas were mostly packed with open forest and modest density forest. The terrain is generally flat. Nagpur division is surrounded by Many National parks and Sanctuaries. Wolves are primarily found in the buffer areas of these protected areas. Co-predators in those stretches are tigers (*Panthera tigris tigris*), Indian leopards, sloth bears (*Melursus ursinus*), striped hyenas, dholes (*Cuon alpinus*), and golden jackals. Prey species are sambar (*Rusa unicolor*), nilgai (*Boselaphus tragocamelus*), chital (*Axis axis*), chousingha (*Tetracerus quadricornis*), and wild pigs.

Data collection. The howls from the Indian wolves were recorded from November 2015 to July 2016. The howls were recorded during the systematic howling surveys accompanied by the opportunistic and spontaneous recordings of captive and free-ranging wolf howls. Howling surveys were done in the early morning (from 4:30 am onwards) and early evening hours (up to 7:45 pm) [time varies depending on sunrise and sunset]. The survey protocol was adapted from Harrington and Mech[60]. Each howling session consisted of five trials with

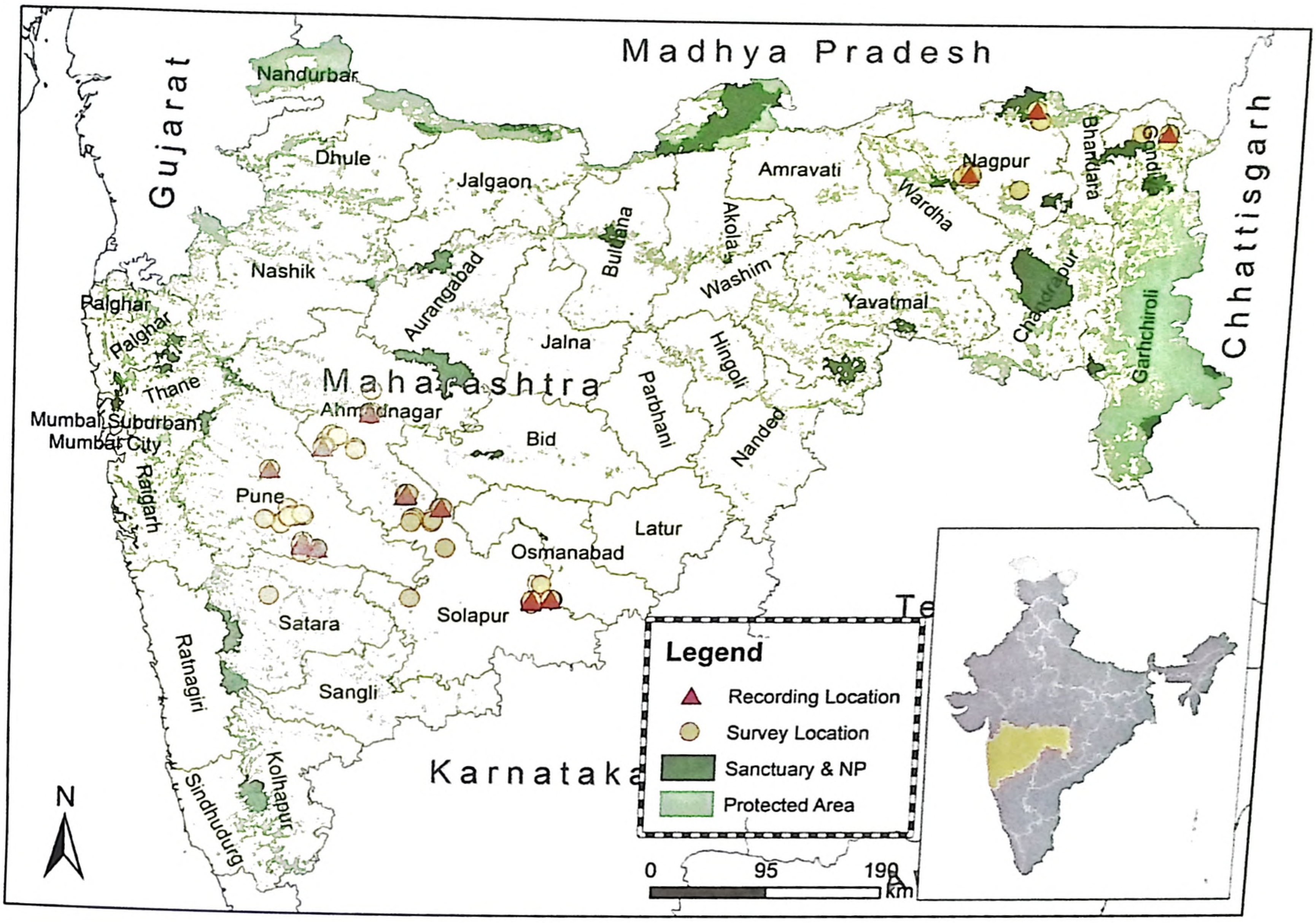

Figure 1. Map showing howling recording locations of the free-ranging wolf in six districts of Maharashtra. Yellow round bullets indicate the survey locations and Red triangular bullets represent the howling recording sites.

three-minute intervals. A series of 50-s-long pre-recorded solo howls (from an individual in Jaipur Zoo) was played three times with increasing amplitude; the session was followed by a 50-s-long chorus howl (from three individuals in Jaipur Zoo) in the order of mid and highest amplitude level of the speaker respectively. A 40-W JBL Xtreme speaker (Harman International Industries, 2014) was used for the playbacks. If howling responses were recorded, the playback session was terminated and repeated after 15 to 20 min. All howls were recorded in a single microphone setup, using a Blue Yeti Pro USB Condenser Microphone (Blue Microphone, 2011) attached with Zoom H4N Handheld Audio Recorder (Zoom Corporation, 2009) with a sampling rate of 44.1 kHz and 16-bit depth.

Ethical approval. The study on captive wolves in zoos was done with the permission of the Director of Jaipur Zoo and the Forest Department of Rajasthan, India [Letter no- 3(04)-II/CCFWL/2013/4586–87; Dated 30th Oct 2015]. The survey of free-ranging wolves of Maharashtra was performed with the consent of the Principal Chief Conservator of Maharashtra Forest Department [Letter no- 22(8)/WL/CR-947(14–15)/1052/2015–16; Dated- 6th Aug 2015]. No animal was harmed during the study, and the standard non-invasive protocol of howling survey was maintained. All the data collection were approved by the Animal Ethics committee of Wildlife Institute of India, Dehradun, India.

Feature extraction. The howls were sorted, and spectrograms were generated using a *Discrete Fourier Transform* (DFT) algorithm in *Raven Pro 1.5 software*[61]. *Discrete Fourier Transform* (DFT) algorithm transforms the same length sequence of equally spaced sample points (N, where N is a prime number) with circular convolution being implemented on the points[62]. All the spectrograms were produced using *Hann windows* at the rate of 1800 samples on 35.2 Hz 3 dB filter (Fig. 2). Only recordings with low levels of background noise and without any overlapping sounds, where the howls were clearly visible as contours, were selected for further analysis. Spectral images were digitised using *Web Plot Digitizer Software*[63]. Thirteen different features (Table 1) were measured from the digitised value by using Microsoft Excel. The details methodology is represented in Fig. 3.

One hundred and thirty-three howls that were longer than 5-s were used for further analysis, with more than ten individual wolves included. The 5 s cut off were chosen to avoid social squeak calls that are very similar to

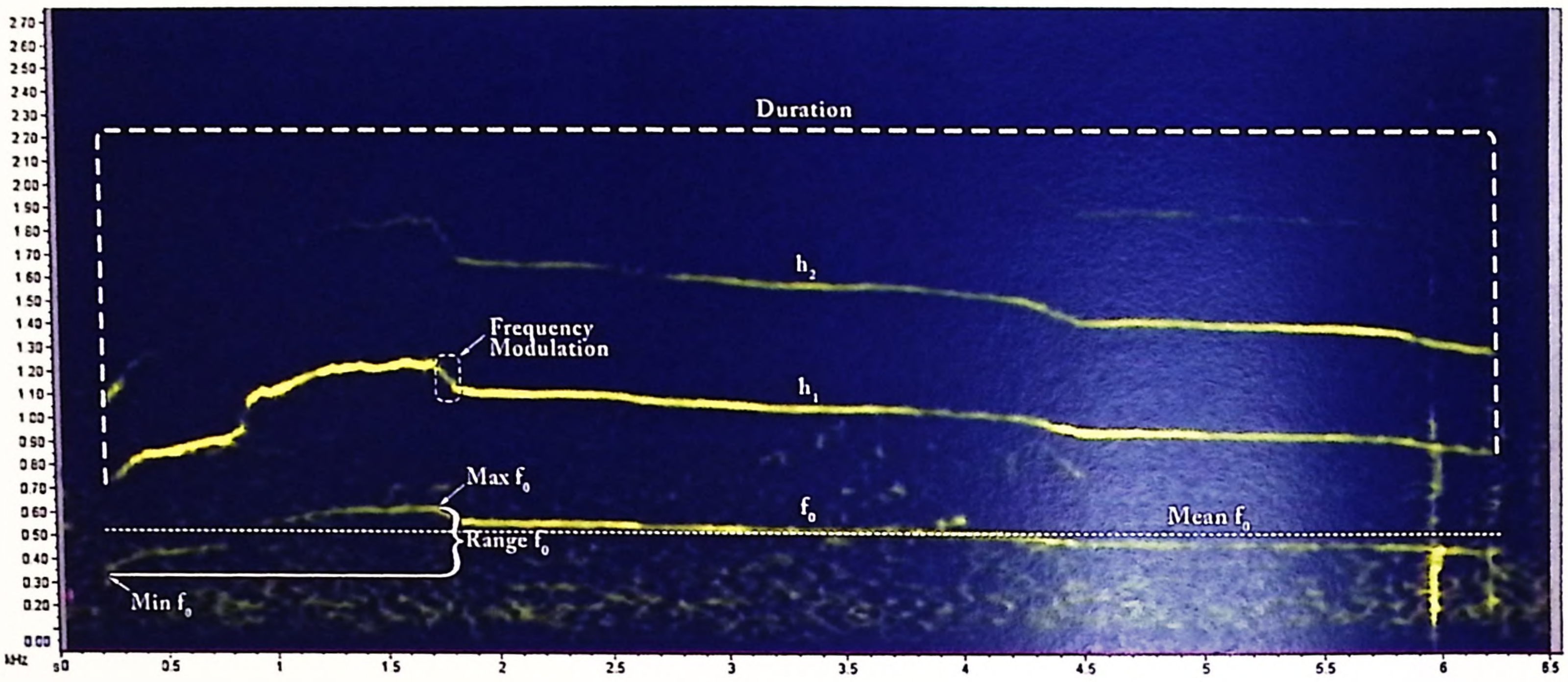

Figure 2. Spectrogram of Gangewadi Wolf howl (160203-001_Gangewadi2_A5) showing how different variables were measured.

Variable name	Definition of variable
Min f	The minimum frequency of the fundamental (f_0)
Max f	The maximum frequency of f_0
Range f	Range of f_0; f_0 = Max f – Min f
Mean f	Mean frequency of f_0 at 0.1 s interval over the duration
Duration	Duration of Howl measured at f_0; Duration = t_{end} - t_{start}
$Abrupt_{0.025}$	Number of abrupt changes in f_0 more than 25 Hz at single time step (0.1 s)
$Abrupt_{0.05}$	Number of abrupt changes in f_0 more than 50 Hz at single time step (0.1 s)
$Abrupt_{0.1}$	Number of abrupt changes in f_0 more than 100 Hz at single time step (0.1 s)
Stdv	Standard deviation of f_0
Co fm	Coefficient of frequency modulation of $f_0 = \Sigma\lvert f(t)-f(t+1)\rvert/(n-1) \times 100/\text{Mean } f_0$
Co fv	Coefficient of frequency variation of f_0 = (SD/mean) × 100
Pos Min	Position in the howl at which the minimum frequency occurs = (time of Minf)/Dur
Pos Max	Position in the howl at which the maximum frequency occurs = (time of Maxf)/Dur

Table 1. Thirteen different variables that were measured from the fundamental frequency (f_0) [Lowest frequency of periodic waveform of each howl].

howl but shorter ($\bar{x}$ = 3.87 s) and high-frequency variable calls, described by Sadhukhan et al.[55]. Also, the longer howls might contain more identification features than the shorter howls do. *Principal Component Analysis* (PCA) was conducted on measured parameters of 133 howls to reduce the dimension and emphasise the variation between each howl. Out of 133 howls, only 69 howls were identified to an individual. The 69 howls were from nine wolves with known identities: three were captive wolves and six wild, free-ranging wolves, which were identified from their visual features when they were howling in front of the observer and thus howls could be attributed to them individually. The data was further subdivided into training and test datasets. Forty-nine howls from five individuals (2 captives; 3 wild) were used as the training data, and 20 howls from four different individuals (1 captive, 3 wild) as test data to ensure the validity of the method (Table 2). Since the known wolf howls were used test data never used in building model, it provides 'unbiased sense of model effectiveness'[64].

Discriminant function analysis. Linear *discriminant function analysis* (DFA) was performed with 49 howls from five individuals (training data) using seven PCA values that contributed more than 5% variation (Table 3) [The cut off value was chosen from scree plot, See Supp. Material 1: PCA.pdf]. The objective of DFA was to construct the linear combination of independent *principal component variables* (PC1–PC7) that will discriminate howls of different individuals. The howls were plotted with discriminant functions at two-dimensional space followed by the group prediction (Fig. 4).

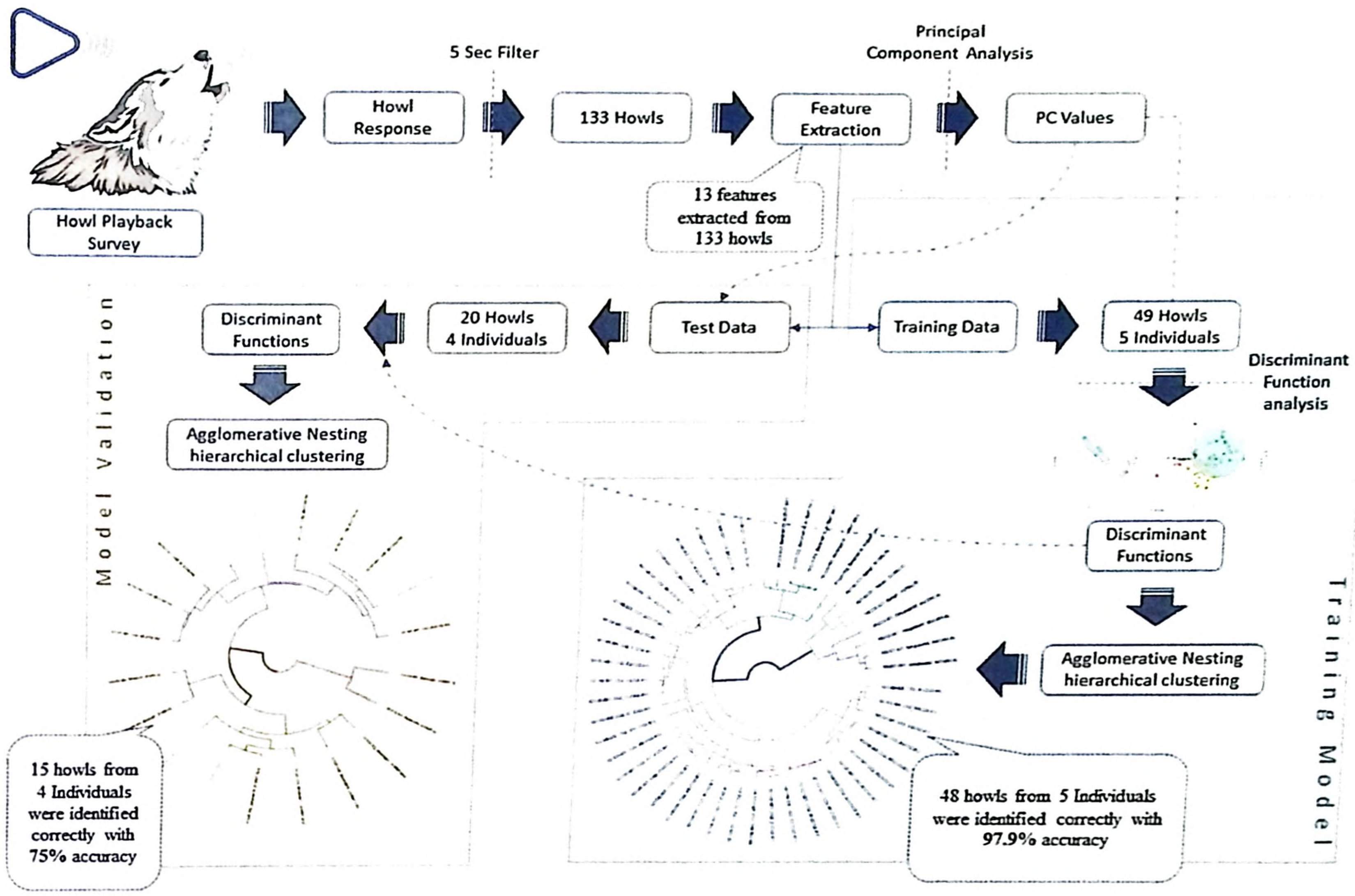

Figure 3. The pictorial representation of methodology for identifying unknown Indian wolves by their howls.

Training/testing	Wolf name	Captive/wild	Capture date	No. of howl
Training data (n = 49)	BMT.SA1	Wild	20/12/2015	5
	CG1.A1	Captive	06/11/2015	3
			08/11/2015	6
	CG2.A1	Captive	05/11/2015	8
			07/11/2015	11
			08/11/2015	9
	GWD.A	Wild	03/02/2016	4
	NNJ.A	Wild	30/01/2016	3
Test data (n = 20)	BMT.A	Wild	19/12/2015	4
	BMT.SA2	Wild	20/12/2015	4
	CG2.A2	Captive	07/11/2015	7
	NU.A	Wild	28/04/2016	5

Table 2. Table showing the information on each individual wolf and their capture date with the number of howls were used in this analysis.

Hierarchical clustering. To test the success rate of identifying different individuals from their howls with *Linear Discriminant* (LD) score, an *Agglomerative Nesting hierarchical clustering* (AGNES) was executed on 49 howls (training data) that were used in DFA. AGNES initially considers each howl as a different cluster and use a '*bottom-up*' algorithm to join different clusters based on the similarities[65]. The analysis was performed in R using '*agnes*' function in the package '*dendextend*' and '*manhattan*' metric was used to build the cluster[66]. The same analysis was performed on the test data to determine the accuracy of identifying unknown individuals and estimating the number of wolves from their howls. While the test data contained howls from known individuals, the wolves' identities were not included in the model. The variables of these 20 howls were calculated from the equation of DFA of 49 howls for cluster analysis.

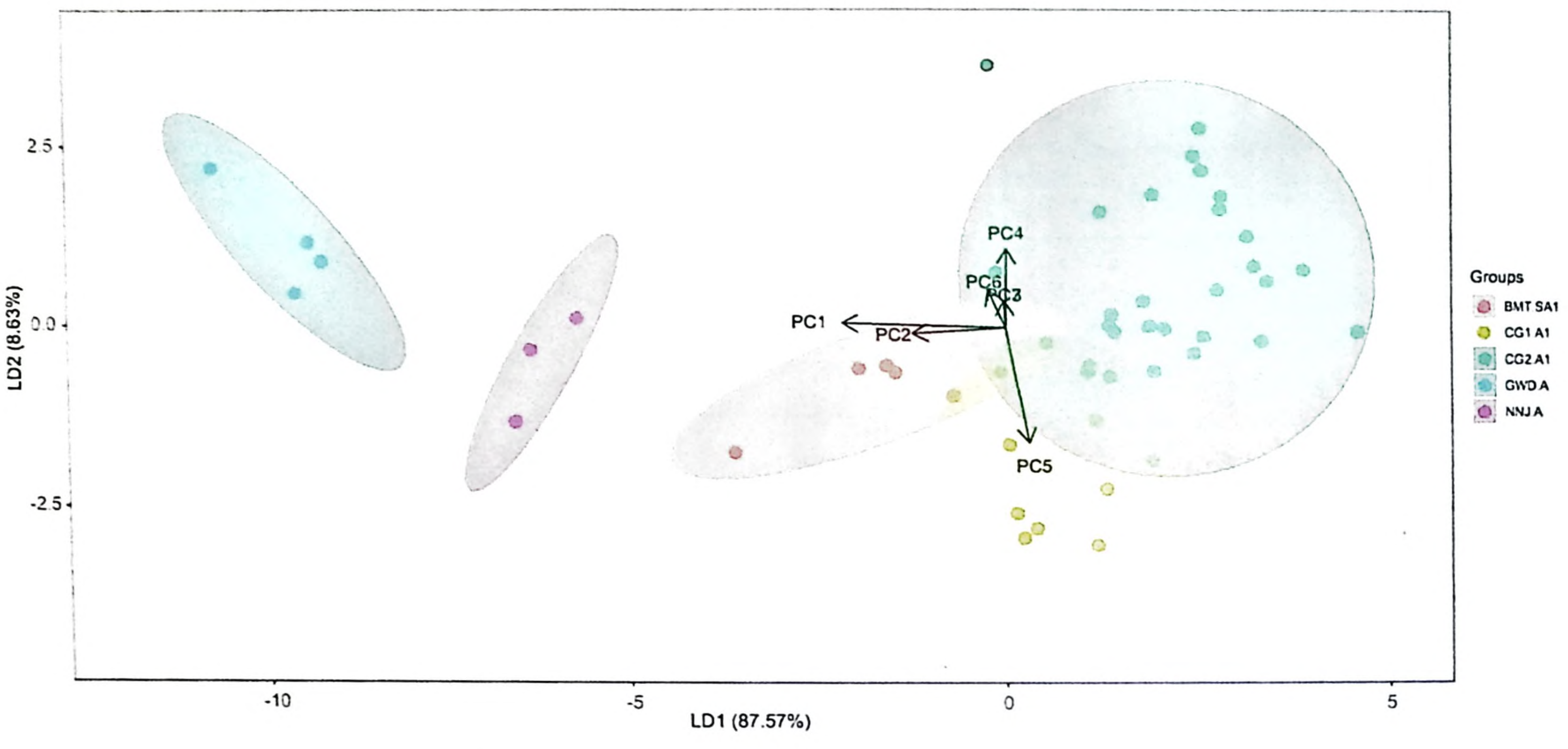

Figure 4. Figure showing a two-dimensional plot of discriminant function analysis using LD1 (Linear Discriminant) and LD2. Each colour represents each wolf. 100% accuracy was achieved in identifying 49 howls from five Indian wolves.

	Component importance (%)
PC1	**41.2**
PC2	**16**
PC3	**10.5**
PC4	**8.1**
PC5	**6.8**
PC6	**6.5**
PC7	**5.7**
PC8	2
PC9	1.7
PC10	1.1
PC11	0.4
PC12	0
PC13	0

Table 3. Table showing the percentage of variation each principal component (PC) accounts for first seven PC function (marked as bold) contributed 94.8% in describing the variable.

Results

Dimensions reduction to emphasis on variation among howls. Seven *Principal Components* (PC) that explained more than five percent of the variance (Table 3) each were generated from 13 scalar variables (Table 1). These seven PCs together explained 94.8% variance among different howls (Fig. 5). SD of the fundamental frequency (f_0), Frequency (f_0) range, Maximum f_0 and the number of abrupt change (> 25 Hz) were the most important contributing factors for building PC1 which contributed 41.2% explaining the variable (Fig. 5).

Building discriminant function to emphasis on howl variation among different individuals. The objective of DFA was to build an equation that discriminates the howls of different individuals. The LD score also highlights the variation among howls from different individuals. DFA achieved 100% accuracy in identifying five individuals from 49 howls (Fig. 4). As the first two *Linear Discriminants* (LD1 and LD2) were responsible for 96.2% of the variance to differentiate between howls of different individuals (LD1 = 87.57% and LD2 = 8.63%), we calculated LD1 and LD2 for rest of the howls using the same function (equation) from last DFA. PC1 and PC2 contributed 85% in building LD1; PC4 and PC5 are the most crucial factor (65%) for LD2 function (Fig. 5).

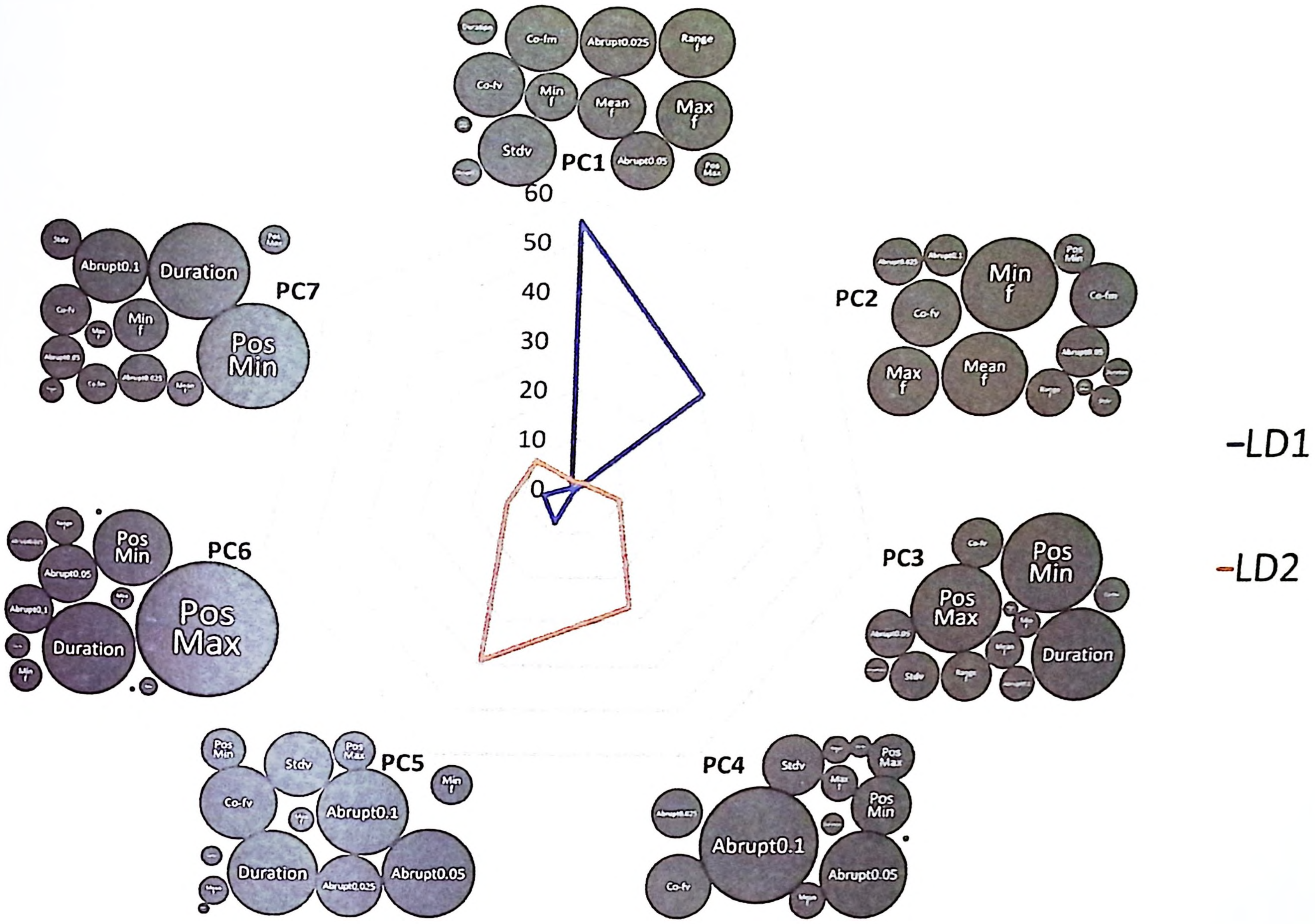

Figure 5. The spider web bubble plot is describing how the *Simple Scalar Variables* (SSV) are ultimately contributing to two LD functions through PC values. The bubble size of each SSV represents the contribution for building each PC function. The blue line represents LD 1, and Orange represents LD2. Since PC1 and PC2 contribute 85% for LD1, the most important SSVs are Stdv f_0, Min f_0, Max f_0 and Mean f_0. Similarly Duration, Abrupt changes, Co-fv contribute the most in building the LD2 function via PC4 and PC5. LD1 was best defined by the different fundamental frequency factors, while LD2 was best defined through the shape of the frequency contour. Therefore, the critical factors for individuality were encoded in X and Y variables.

Identifying individuals from their howls in testing dataset. First, we tested AGNES on the training dataset (49 howls from 5 individuals) and found 48 howls (~ 97.9% accuracy) were identified correctly at 2.2 clustering scale (Fig. 6). When the same analysis was performed on 20 howls of four different individuals to test the accuracy for the non-training dataset, 15 out of 20 howls from (75% accuracy) four individuals were identified correctly at 2.2 clustering scale (Fig. 7; Table 4). Two howls from wolf BMT.A were misclassified to wolves BMT.SA2 and CG2.A2; Three howls from wolf NU.A were misclassified to wolves BMT.SA2 (1 howl) and CG2.A2 (2 howls) (Fig. 7; Table 4).

Discussion

Here, we presented a new approach to train the classification model, which can identify individuals from their howls and determine the number of wolves present in a certain number of howls, allowing for fine-scale population surveys. In this study, we built an identification model with known training data which was verified with novel test data. The testing data included howls from the known individuals of both captive and wild Indian wolves but independent from the training dataset so that we can cross-check the identification accuracy without bias. The key finding of our study was 97.9% wolf howls were identified correctly from training data, whereas the accuracy of the model on the testing data was 75%. Moreover, we were able to identify four individuals accurately from the testing dataset. The primary significance of this study is that it can be replicated for any other wolf sub-species with a set of a known wolf howls. This study increases the feasibility of wolf pack census using a howling survey[15,60]. Since wolves may actively avoid camera traps[54] and photo-identification of wolf requires arduous effort[3,67], identifying wolves from their howls is a big step towards population estimation using CMR.

Although CMR associated with camera trapping provides population estimation without bias for an identifiable animal like a tiger[68], camera trapping has several limitations for non-identifiable and long-ranging species like the wolf[1]. Other non-invasive methods like DNA-based CMR resulted in biased population estimation due

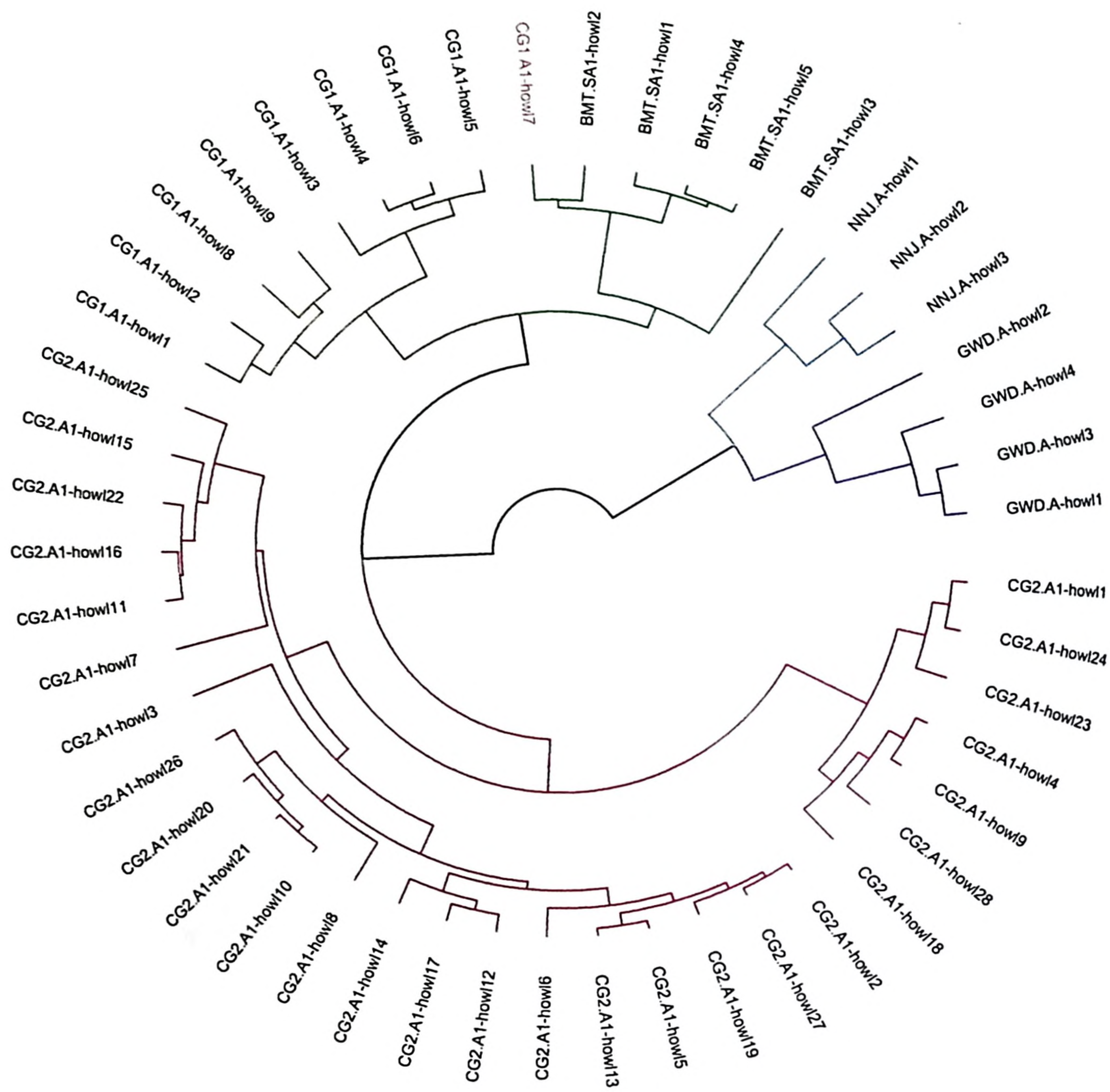

Figure 6. Hierarchical Clustering of 49 howls from five individuals. These 49 howls were used in training the data. 48 howls were identified correctly with the accuracy of 97.9%. The wrongly identified howl is marked in red.

to the animals' non-uniform scent-marking patterns[59,69]. However, acoustics based surveys allow vast area sampling with limited resources as compared to camera trapping and other non-invasive methods[3]. Furthermore, our field observations of wolves have shown that the whole pack typically howls during choruses and that all individuals are acoustically active.

For population size estimation through an acoustics-based survey, a combination of CMR and Distance Sampling is required to reduce bias and heterogeneity in detection probability[27,70]. Identifying individual wolves from their howls close this gap of implementing the CMR technique for the population assessment of this elusive and challenging to track species[7,25,27]. While a few studies have established that howls carry individuality information[34] and known howls can be distinguished from each other[39,13,71], no study has been successful before in identifying unknown individuals from a set of howls. Furthermore, attempts to count the number of individuals present in a recording have been limited by difficulties in minimising confidence intervals[18,72]. There are two ways to identify individual wolves or packs—supervised clustering and unsupervised clustering. While supervised clustering requires a set of known training data and cluster validation is straightforward, unsupervised clustering requires ground-truthing before it can be used to monitor populations at a survey level and does not allow individual level CMR or tracking[30].

Although DNA-based identification from faecal sampling is more accurate in identifying individuals than our result, it has drawbacks, such as biased population estimation and the increased cost and effort required to collect and analyse the faeces[59,69]. Nevertheless, the acoustics-based identification model requires further work to increase its accuracy, though we believe that the successful implementation of this method as a CMR-based supervised population estimation model is already possible.

Wolves mostly live in packs that habitually howl together, and it is challenging to identify the specific wolf that is howling, particularly in choruses. If included and incorrectly attributed to a particular wolf, these howls could

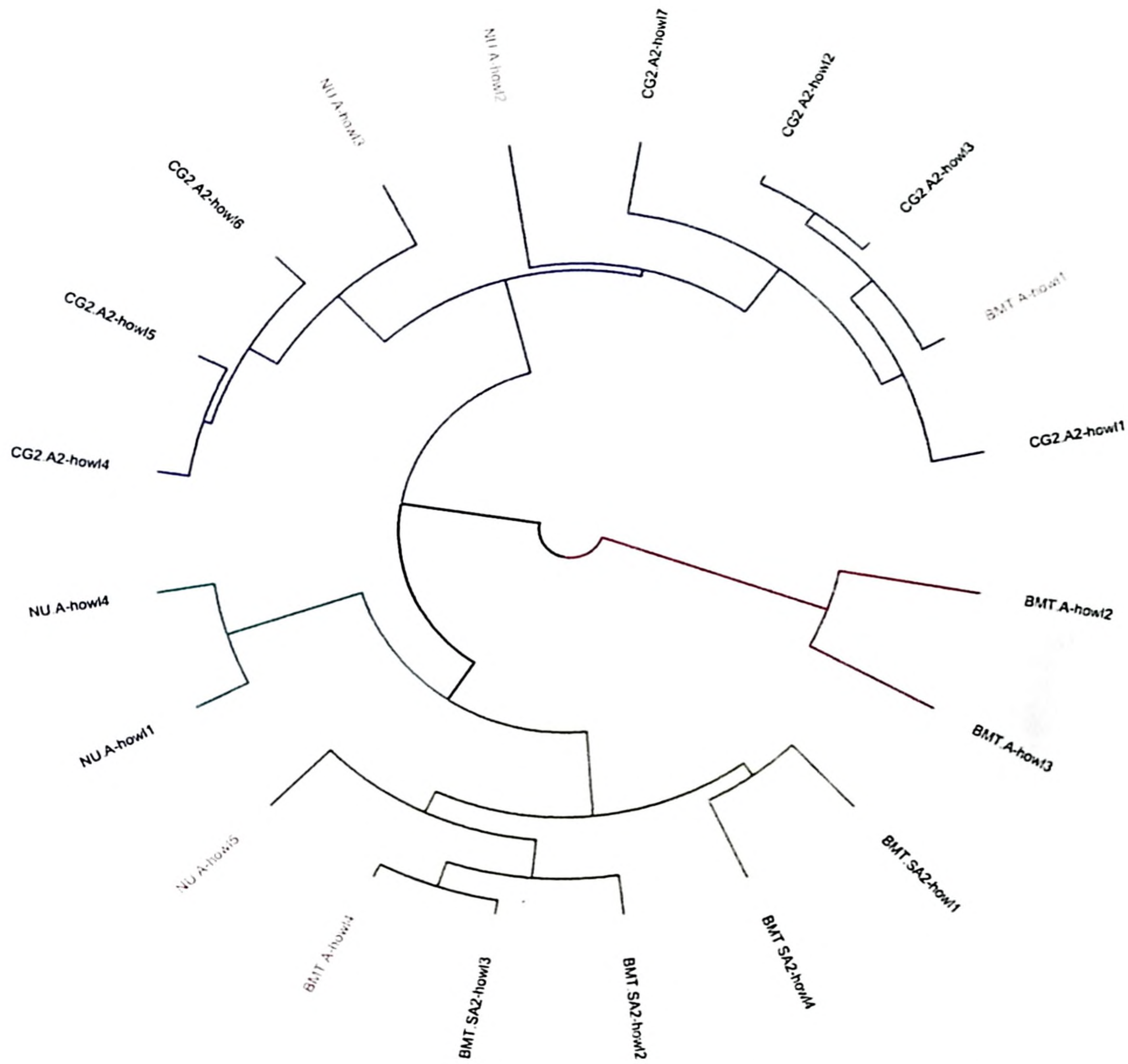

Figure 7. Hierarchical Clustering of 20 howls from four Indian wolves. None of the 20 howls was used in training the data. 15 howls were identified correctly with the accuracy of 75%, and all the four individuals were identified correctly as different clusters. The correctly identified howls are marked in black, and the five wrongly identified howls are marked in red.

	Predicted group membership					
Individuals	**BMT.A**	**BMT.SA2**	**CG2.A2**	**NU.A**	**Identification accuracy (correct/total)**	**Total**
Count						
BMT.A	2	1	1	0	2/4	15/20
BMT.SA2	0	4	0	0	4/4	
CG2.A2	0	0	7	0	7/7	
NU.A	0	1	2	2	2/5	
Percent						
BMT.A	50	25	25	0	50	75%
BMT.SA2	0	100	0	0	100	
CG2.A2	0	0	100	0	100	
NU.A	0	20	40	40	40	

Table 4. Details of individual identification accuracy using hierarchical clustering on testing data (20 howls from four individual). 15 out of 20 howls were identified correctly with the accuracy of 75%.

lead to erroneous predictions by the model. Therefore, this limited our potential data set to those howls which were conclusively attributed to a known individual, and we dropped many howls, especially the chorus howls, from the analysis to avoid misleading the model. However, larger training datasets from different wolf populations might increase the efficacy of the identification model and verification with more wolf howls conceding better reliability as found for Southwestern Willow Flycatcher[73]. Thus, our result of 75% may represent a baseline, not a limit, on the accuracy we could achieve. The inclusion of multiple series of howls from every individual would give a more precise result. However, since none of the free-ranging wolves was radio-collared or marked, this was not possible for the wild wolves. Studying howls of collared wolves would help in adding multiple howl sequences from many free-ranging wolves in the training data and may fill this research gap.

This study revealed that the number of wolves present in the recordings could be determined from their howls and the individuality information is sufficient for supervised population estimation through CMR techniques[7,25,27,30]. Therefore, wolves recorded in one location can be acoustically recaptured at another location, and we can identify them individually. Since our model is exclusively built on fundamental frequency, changes in terrain or vegetation should not affect the accuracy of the model. The information gained from recapturing wolves across different locations would help in deriving territoriality (home-range) information, and this information is crucial for spatially explicit individual-based point process models. This is a clear advancement for developing howling playback surveys as a wolf pack census method. Regular population monitoring will help towards conserving and saving this cryptic species before its population falls beyond a recovery level. Furthermore, since wolf howls can be detected across distances of more than 6 km, identifying wolves from their howls also opens up a new opportunity for non-invasive tracking of this species across large landscapes.

Guidelines to implement the methodology on the field. We used this methodology to identify individual Indian wolf howl. However, one can use this methodology to identify species, sub-species or individual from their calls. This requires a set of calls to make up the training dataset and a set of calls to make up the testing dataset. We recommend some precautions and step by step guidelines for adapting this method.

I. Before the data collection, one should be cautious about choosing the recorder and data collection methodology. Although we are not definite about the impact of multi-recorder setup in identification accuracy, we recommend using a single microphone set up to keep consistency, especially for individual identification as differences in sensitivity and recording parameters can influence acoustic integrity [See[45]].
II. The multiple groups in the training dataset should be carefully selected to represent distinct group member calls with high confidence (e.g. species/sub-species/individuals), as a single incorrectly identified call in the training dataset can lead the model to erroneous results.
III. The selection of appropriate spectral features is important. While many species encode their identity in the same features, some encoding is species-specific. We tested a wide range of software which fell short in feature extraction for overlapping calls or where background noise was present. The feature description is only as reliable as the extraction. Here, we used *web-plot digitiser* software for spectrogram digitisation. We recommend the use of any semi-automated graph digitiser tool for noisy or overlapping spectral data.
IV. The training data should contain only known groups (multi-species/multiple sub-species/multiple individuals). Each training group should have at least three to five calls and recordings from multiple sessions will increase the accuracy of the model as the animals may have higher intra-individual variation across days than within them. Thus, the higher the intra-individual or intra-group variation, the greater the number of vocalisations and individuals that should be included in the training dataset to make a robust model for the testing dataset.
V. Even though one can choose an unknown dataset as test data, we recommend using a known dataset when originally validating the model. Using multiple test datasets will increase the model's confidence.
VI. We recommend using multiple small batches as test data (50–100 sample of calls) instead of large data to avoid confusion in cluster groups that may represent other variation in the calls.
VII. To allow study replication, we have made our data and codes available in the Supplementary Materials. While the data needs to be replaced for each study, the system of analysis and classification should be robust and replicable.

Conclusion

Our study reached substantial accuracy in identifying wolves from their howls. Since the methodology was validated using known wolf data and was found to be reasonably reliable, unknown howls can also be classified. This opens up a new opportunity for population estimation and tracking of wolves through howling surveys. Although we analysed our data with Indian wolf howls, the procedure is replicable for other subspecies that have a set of known howls from different individuals and could potentially be applied to other species with individually distinctive vocalisations. This would refine and improve both population estimates and the ability to monitor individuals in situ, with global implications for conservation and ecology.

Appendices: Supplementary materials

All the data and R code require to recreate the analysis are hosted in https://github.com/bhlabwii/wolf_howlID platform. Raw sound files are available on request to the corresponding author. Compiled reports from R Scripts can be found in following supporting material:

Filename	Description
PCA.pdf	Principal Component Analysis of 133 howl
DFA.49H5ID.PCvalue.pdf	Discriminant Function Analysis of 49 howls from five individuals
known_dend_49H5ID.pdf	Agglomerative Nesting hierarchical clustering (AGNES) using 49 howls from five individuals

Filename	Description
Dendrogram.test.pdf	Agglomerative Nesting hierarchical clustering (AGNES) using 20 howls from four different individuals to test the model

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Author contributions

B.H. conceptualised the study. S.S. collected all the data and did data extraction, analysis and writing the manuscript. H.R.G. and B.H. both supervised in data interpretation along with the manuscript writing. B.H. played a sole role in funding acquisition. All authors contributed critically to the drafts and gave final approval for publication.

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Wildlife Institute of India

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This is to certify that

Sougata Sadhukhan

presented a paper during the 12th Internal Annual Research Seminar held on 26th September 2016 at the Wildlife Institute of India.

In recognition thereof, this **Certificate of Participation** is hereby awarded on this 30th day of September, 2016 at Dehradun.

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from Saturday 31st August to Thursday 5th September 2019,

Signed:

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On Behalf of the IBAC 2019 organising committee

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Sougata Sadhukhan

participated and presented paper/poster paper in International Conservation Conference
held at Aligarh Muslim University, Aligarh, U.P., India
during 21st to 23rd October 2019. The title of the presentation was

Characterising the Harmonic Vocal repertoire of the Indian Wolf

DR. AFIFULLAH KHAN
Conference Director

Orus Ilyas
DR. ORUS ILYAS
Conference Co-ordinator

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