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Microbial activity in the cold and dry High Himalayas

Regeneration of Pinus wallichiana

New record of Malaxis biaurita for Nepal

Climate change and disease risk in the Himalayan region

Overuse of agricultural pesticides in Nepal

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Climate change and disease risk in the Himalayas

Climate change is likely to increase the burden of infectious diseases in the Himalayas; systematic preparation must start now

Sahotra Sarkar

The International Centre for Integrated Mountain Development (ICIMOD, Kathmandu), with funding from the MacArthur Foundation, has recently performed a detailed analysis of the impacts of climate change on the Eastern Himalayas (Sharma et al. 2009). Their report focuses primarily on biodiversity and ecosystem services. As expected, the predictions are dire while the uncertainties remain large, given the paucity of serious attention to this region compared, for instance, to the Alps or even to the Andes. While the report notes the implications of climate change for human well-being, including health, infectious disease gets only passing mention (three paragraphs out of 25 pages). This is unfortunate because there is ample reason to expect that climate change in the Himalayas will lead to a significant increase in the regional infectious disease burden due, in particular, to vector-borne tropical diseases that have historically been absent from the Himalayas.

A new challenge is unfolding in the Himalayas: a significant increase in the burden of infectious disease, driven by climate change. Vectors are moving beyond their historic ranges to higher elevations; water quality is deteriorating, and the available supply is diminishing. Preventive and ameliorative measures to address these problems require robust quantitative estimates of the size and spatial distribution of disease risk. Once enough data are available, disease risk can be mapped with predictive models so that appropriate policies can be formulated and implemented. Unfortunately, there has been virtually no quantitative epidemiological attention to this region.

The reasons are simple. First, climate change is likely to lead to a deterioration of water quality, partly because the increased initial meltdown and excess in water availability will be followed by a very significant reduction in supply. Resulting shortages of good quality water will increase the burden of diarrheal disease. Even cholera may emerge as a significant problem because of its known association with climate (Colwell 1996), and given its current incidence in some parts of the Indian Himalayas and Nepal. Second, there will also be a likely decrease in air quality, with a higher concentration of pollutants such as nitrogen dioxide and airborne-particles (the latter primarily in urban areas), and an increase in lower tropospheric and ground-level ozone levels, all leading to an increased frequency of cardio-respiratory disease.

Third, and perhaps most important, systematic temperature increases (0.01–0.04°C per year, according to the ICIMOD scenarios) will allow tropical diseases to expand their range to higher elevations at which they did not occur before. These include a large spectrum of vector-borne (and, often, reservoir-dependent) diseases that have already begun to become problems in the region, including (visceral) leishmaniasis (locally known as *kala-azar*), dengue, and malaria. (The ICIMOD report also mentions schistosomiasis but it is unlikely that this disease will emerge as a major problem in the Himalayas given its very low frequency in neighboring regions.)

The possible upward spread of leishmaniasis is particularly worrisome because it has recently recently begun to be established at an altitude greater than 600 m in Garhwal and Himachal Pradesh where it was previously not endemic (Raina et al. 2009). The implicated vectors are almost certainly Phlebotomus or Sergentomyia species which have ranges restricted by climate. Range shifts of these vector species to

Aedes aegypti, the principal vector of dengue, has already advanced upward from its historic range.

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higher elevations may have already been induced by climate change, as suggested by theoretical analyses of the climatic dependencies of these species (Cross et al. 1996, Kuhn et al. 1999, González et al. 2010). Similarly, clinical cases of dengue, as well as larvae of its principal vector, *Aedes aegypti*, have recently been recorded in the Kumaon hills at elevations higher than previously reported (Shukla and Sharma 1999). In the case of falciparum malaria, there is documentation of its spread into higher altitudes in the Himalayas (Bouma et al. 1996, Bhattacharya et al. 2006). Himalayan populations, with no prior history of exposure to these pathogens, are likely to be more vulnerable than their tropical counterparts.

It is critical that concerted action be taken to prepare for these problems and to investigate if other diseases are also likely to expand their range due to climate change. Unfortunately, except for malaria (the spread of which has been investigated for all of India), there has as yet been no systematic analysis of the emergence of previously absent diseases for any region of the Himalayas. Partly, this reflects the general problem that these other tropical diseases associated with poverty have largely been ignored by recent medical research (Hotez et al. 2007). But it also reflects a lack of quantitative epidemiological and ecological attention to the Himalayas.

Nevertheless, quantitative predictive models of disease risk are necessary prerequisites for any health planning for the region: not only must these models predict the extent of the risk for each specific disease, they must also predict the spatial distribution of this risk. Otherwise, efficient allocation of limited resources to prepare for the future—that is, where and to what extent preventive and ameliorative medical materials and expertise should be distributed—will be impossible. Right now, high resolution risk profiles for disease are not available for a single Himalayan region. Moreover, georeferenced disease incidence data, organized by date, which are necessary for any analysis, have never been collated. Basic data availability for the Himalayas lags behind even tropical Africa.

It is likely that the data availability problems can be relatively easily solved, and the necessary analyses performed, for some regions, including the Indian Himalayas and Tibet. However, for other regions, including Bhutan and Nepal, basic epidemiological information is typically lacking for the implicated diseases, partly because these diseases are rare and, therefore, often unrecognized. Even the World Health Organization's regional strategy shows a lack of urgency and entirely ignores the new epidemiological challenges posed by climate change (WHO 2007). But, without immediate and sustained attention to these issues, the challenges will not be surmounted.

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Mancozeb: growing risk for agricultural communities?

The short- and long-term health consequences of pesticides are real

Kishor Atreya and Bishal K Sitaula

In a country where farmers rely on conventional wisdom to make decisions on farming practices and the government lacks clear policies based on solid research, pesticide overuse is emerging as a problem. Mancozeb, the widely applied pesticide in Nepal's vegetable farming, has both short- and long-term health consequences to people exposed to its unsafe levels. A handful of studies in the hill regions of Nepal suggest that the pesticide is being sprayed to farms at much higher level than recommended. The widespread misuse and dangerous consequences of this pesticide suggest a need for more thorough study, better instruction, and more effective control.

Since the 1950s, when DDT was introduced in Nepal for the purpose of malaria control, many other pesticides have been registered for use. Chemical pesticides are used by 25% of Terai households, 9% of mid-hill households, and 7% of mountain households (CBS 2003). In certain mid-hill pockets close to urban markets, the penetration of pesticide use is considerably higher. The incorporation of vegetables into Nepal's cereal-based agricultural production system, especially in the hills, has stimulated a significant demand for chemical inputs such as pesticides. Although pesticide import declined after 2002, that trend has apparently reversed, with import substantially increasing in 2007 and 2008 (**Table 1**). Assuming all the imported pesticide is consumed, the pesticide use at national level for the year 2008 was 151.2 g active ingredient per ha of arable land (total arable land = 2,357,000 ha; UN [2010]).

Chemical pesticides are known to have deleterious effects on human health and on the environment. A series of studies (e.g., Dahal 1995, Pujara and Khanal 2002, Atreya 2007a, b, c, 2008a, b), highlighting the massive use of chemicals in vegetable growing areas has raised the issue of possible health risks for our agrarian communities. Studies conducted abroad have linked mancozeb, a synthetic pesticide, to serious hazards including cancer. Now we have evidence that farmers are applying mancozeb at levels significantly exceeding the manufacturer's recommendations (Atreya 2007c, 2008b).

Mancozeb, a grayish-yellow powder, is used to control fungal diseases that afflict many important economic crops, including potato, tomato, fruits and flowers. It is a broad-spectrum pesticide that indiscriminately kills a range of organisms, targeted as well as untargeted (and beneficial) species. It acts by disrupting lipid metabolism. It has low volatility at standard temperatures and pressure but can be found associated with air-borne particulates or as spray drift. In a moist environment, it hydrolyzes into ethylenethiourea (ETU), ethyleneurea (EU) and ethylene bisisothiocyanate sulfide (EBIS) with a half-life of less than 2 days. In moisture-limited soil conditions, the half-life is 2–7 weeks. The World Health Organization (WHO 2005) classified it as 'non-hazardous under normal use.' Mancozeb enters into body mainly through the skin and from inhalation. Baldi et al. (2006) observed the highest contamination through the hands.

Vegetable farming, an important source of revenue in the hills of Nepal, generally entails heavier applications of pesticides than does cereal farming. In Nepal, a few studies have shown that households apply more than 90% of the total pesticides into vegetable farming. Atreya (2008b) investigated 3637 spray operations performed by 291 households in two VDCs of Kavre district. In 3464 of those operations, mancozeb was used either alone or in combination with other pesticides, making it the most widely used of the sixteen pesticides studied.

Due to its low acute toxicity and short environmental persistence, the amount of mancozeb used is increasing worldwide (Colosio et al. 2002), and so far there are no recorded incidents of acquired resistance to the chemical. In Nepal, mancozeb is marketed under various commercial names such as Dithane M-45 75% wettable powder (WP), Kishan M-45 75% WP, Indofil M-45 75% WP. Mancozeb is also marketed in combination with 8% metalaxyl as Krinoxyl Gold 72% WP and Matco 72% WP.

Farmers use mancozeb primarily to control late blights of potato and tomato at 5–7 day intervals, although a study claims that a 14-day interval application at the recommended dose is sufficient for the purpose (Apel et al. 2003). The recommended dose for control of potato blight is 1125–1500 grams of mancozeb dissolved in 750 liters of water per hectare, at regular intervals of 7–10 days (PRMS 2006). This amounts to a concentration of 1.5–2 grams/liter. It is generally agreed that mancozeb is being used at levels exceeding this recommendation. For instance, Atreya (2007c) calculated the

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Table 1. Pesticide's active ingredients import in Nepal ('000 kg)

Year	Insecticides+	Fungicide	Herbicides	Rodenticides	Others++	Total
1999	43.46	54.53	2.68	4.06	3.69	108.43
2000	62.44	102.77	14.94	3.42	12.48	196.06
2001	60.32	75.44	3.26	4.30	2.83	146.15
2002	60.39	90.57	6.84	1.24	18.55	177.59
2003	85.61	55.20	11.24	7.87	16.46	176.37
2004	35.36	97.04	6.39	1.14	14.17	154.08
2005	65.00	47.70	11.03	1.46	6.08	131.27
2006	46.55	74.37	5.70	1.81	2.85	131.28
2007	60.28	237.37	6.57	37.30	5.97	347.49
2008*	105.81	203.39	11.12	31.09	4.93	356.35

+ includes organochlorines, organophosphates, synthetic pyrithroids, carbamates, botanical insecticides, mixed-insecticides, and other insecticides; ++ bactericides, acaricides, bio-pesticides, pesticide used in public health, and others
Source: Various official records of Pesticide Registration and Management Unit, Crop Protection Directorate, Department of Agriculture, Ministry of Agriculture; * Sabitri Baral, Pesticides Registrar, Office of the Pesticide Registrar, Kathmandu, Nepal.

average concentration of mancozeb as applied to vegetable crops in two VDCs of Kavre to be 4.26 grams per liter of water, more than twice the recommended concentration.

Health effects

Although it has been characterized as 'non-hazardous under normal use,' is less acutely toxic, and less persistent in the environment, it degrades into other products, of which ethylethiourea (ETU) is of greatest toxicological concern. ETU has been linked to sperm abnormalities (Cox 1996). It affects the central and peripheral nervous systems and causes endocrine disruption (Solomon et al. 2000). Many studies have confirmed that ETU is carcinogenic – particularly affecting the thyroid (Solomon et al. 2000, EPA 2001), teratogenic (interfering with embryonic development), and a general irritant (WHO 2005).

Nepal Government recommends that the maximum residue limit on food be 0.20 mg/kg food at maximum (CBS 2008). However, Nepal lacks toxicological studies on the mancozeb induced health hazards. A few survey based studies have revealed immediate and short-term effects from various pesticides. Farmers who spray pesticides suffer a range of symptoms. For those involved directly in pesticide applications, the predicted probability of acute illness in the day of operation is 0.41 (Atreya 2007c). Atreya (2008b) found that headache, skin and eye irritation and throat discomfort increase significantly with exposure to fungicides. The study calculates that the likelihood of developing headache and skin irritation after a single pesticide spray operation are 19% and 8%, respectively.

The demand for pesticides in Nepal is likely to increase. A handful of studies show mancozeb to be the most widely used pesticide in Nepal. Studies around the world have documented a range of health hazards associated with it. Because farmers rarely use any kind of protection gear during spray operations, they are likely to expose themselves to unsafe concentration of pesticides. Long-term effects of the pesticide have not yet been studied in Nepal. However, the application of pesticides at levels much higher than those recommended entails serious risk. As most of the vegetables sold by farming families are grown by independent farmers who determine their own protocols for pesticide application without reference to standard recommendations, significantly high residues are likely to be passed on to consumers.

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Microbial biomass and activity in high elevation (>5100 meters) soils from the Annapurna and Sagarmatha regions of the Nepalese Himalayas

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High elevation subnival-zone soils are increasing in spatial extent in the Himalayas due to glacial retreat and grazing pressures. These seemingly barren soils actually harbor significant microbial diversity but have remained mostly unstudied in all of the major mountain ranges of the Earth. Here we describe a preliminary survey of subnival-zone soils and one vegetated high-elevation soil in the Annapurna and Sagarmatha regions of the Nepalese Himalayas. We examined microbial biomass and activity as well as key microclimatic and edaphic variables that may control microbial activity in these soils. Microbial biomass carbon levels were the lowest ever reported for any soil to date, whereas microbial nitrogen and soil enzyme activities were similar to levels measured in previous studies of subnival-zone soils of Peru and Colorado. Our initial studies also indicate that soil water availability is the primary limiting factor for life in these high-elevation soils.

Key words: Microbial, soil, biogeochemistry, subnival, extracellular enzymes, microbial biomass

On the highest mountain ranges of the Earth, between the upper zone of year-round snow and ice (the nival zone) and the zone of continuous vegetation (the alpine zone), exists a stark expanse of seemingly bare rock and barren soils called the "subnival zone" or "mountain desert" (**Figure 1a**; Nagy and Grabherr 2009, Troll 1973). Yet, upon close inspection the subnival zone is revealed to be a landscape mosaic in which soil development and plant colonization are related to local variation in snow pack accumulation. This variation results in a patchy environment with barren soils underlying high snow areas, sparse plant communities in moderate accumulation areas, and further barren soils in wind-scoured locations. When not snow-covered, the soils of this highly exposed environment are subject to extreme fluctuations in temperature, solar radiation, and soil moisture. This effect is particularly pronounced during the fall season when daily soil temperatures can fluctuate over 40°C, with nighttime conditions below freezing and surprisingly hot daytime soil temperatures around 30°C (**Figure 2**). These factors create a harsh environment for subnival zone organisms and result in one of the most barren looking ecosystems on Earth. It is presently unclear if, given enough time, plants can colonize the upper-elevations of the subnival zone. However, extant subnival soils are dominated by abundant and surprisingly diverse microorganisms even in the highest elevation soils sampled to date (Costello et al. 2009, Schmidt et al. 2009).

Due to the dependence on snow accumulation, the area defined as the subnival zone occurs at much higher elevations in drier mountain ranges such as in the Andes and in the inner ranges of the Himalayas than in more humid mountain ranges such as the Alps. The subnival zone of the Bavarian Alps starts at about 2500 m above sea level (masl) compared to 4700–5000 m in the Andes of southern Perú and 5000–5600 m in the Himalayas (Chang 1981, Rawat and Pangtey 1987). Owing to their extremely high elevation and historical inaccessibility, much less is known about the subnival zone in ranges such as the Himalayas than in the comparatively well-studied Alps. Globally, the subnival zone is thought to have expanded downwards in recent years due to overgrazing in the upper alpine zone (Ahmad et al. 1990, Del Valle et al. 1998) and upwards, due to the retreat of glaciers and icepacks at high elevations (Byers 2007), and is predicted to increase over the next century (Zemp et al. 2006). In Nepal, the subnival zone currently occupies about 6% of the land area (**Figure 1b**); yet we know almost nothing about the organisms that inhabit these environments.

It is still unclear how life forms that survive in the subnival zone obtain the nutrients and energy needed to sustain life. Swan (1963, 1990, 1992) contended that life at these extreme elevations subsisted mainly upon aeolian-deposited

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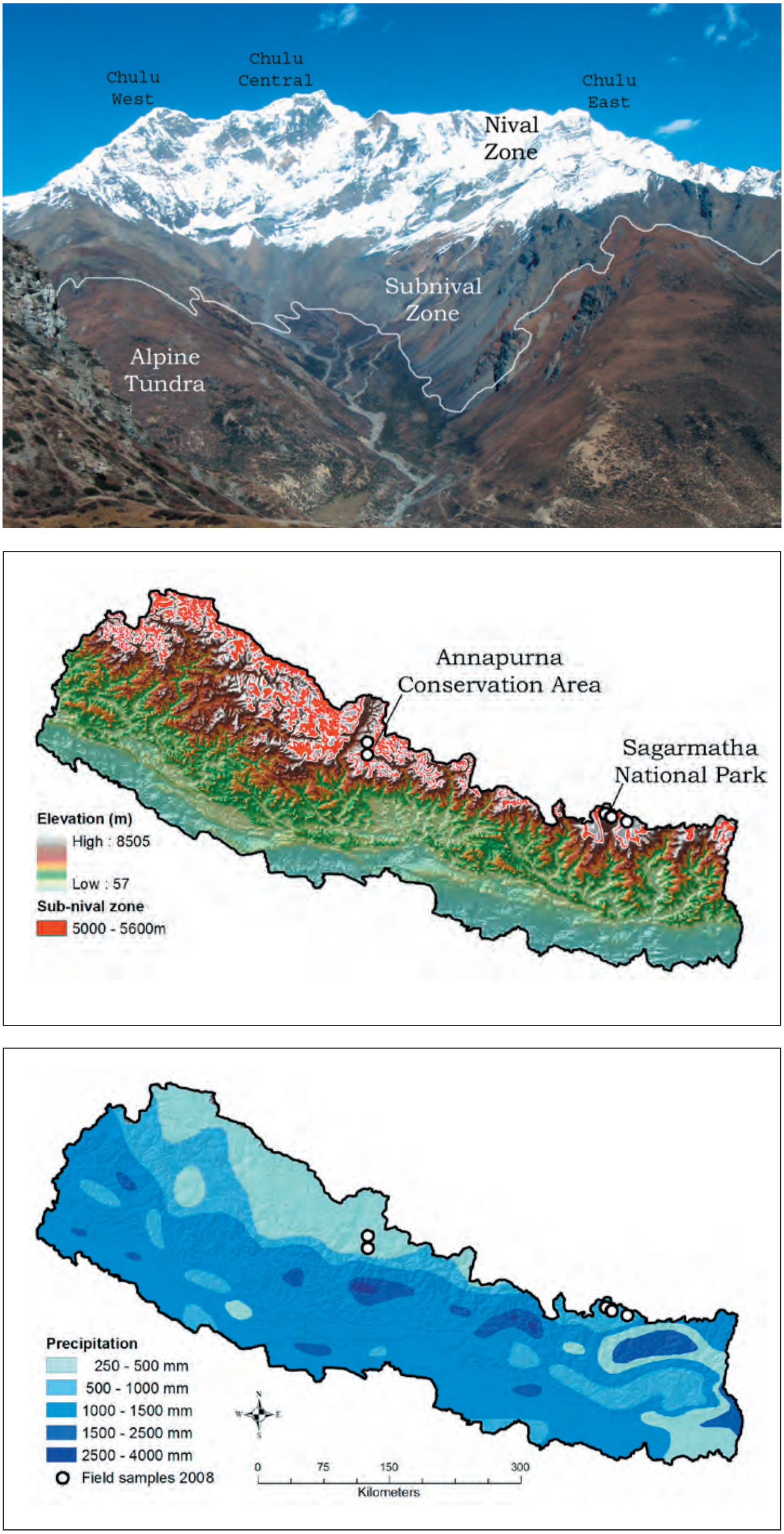

Figure 1. **a (top):** Photograph of the Chulu range taken (10/19/2008) from above Kangshar village during our fieldwork in the ACA. The labels indicate the distinct zonation of the alpine, subnival, and nival zones. The boundary between alpine and subnival zones is defined as the upper extent of continuous plant cover. The boundary between subnival and nival zones is defined as the lower extent of continuous ice and snow cover. **b (middle):** An elevational map of Nepal. The red area represents the extent of the subnival zone as estimated by locations having an elevation of 5000–5600 masl. Sampling locations are in the black circles. **c (bottom):** A map of average yearly rainfall for Nepal showing the sampling sites (data from Nepal Department of Hydrology and Meteorology, http://www.dhm.gov.np).

Table 1. Sampling sites used in this study

Site	UTM coordinates (zone)	Elevation (masl)	Rock type	pH
Zun Tal (ACA)	784700E, 3180600N (44)	5101 – 5289	shale	7.2
Kobresia sward/eroded (ACA)	786770E, 3176125N (44)	4824	soil	6.1
Thorong La (ACA)	787000E, 3188900N (44)	5482 – 5516	shale	7.3
Gokyo Lake 5 (SNP)	468350E, 3097500N (45)	5094 – 5111	granite	5.3
Chola Pass (SNP)	493650E, 3092850N (45)	5376 – 5387	granite	5.3
Island Peak (SNP)	493170E, 3089600N (45)	5272 – 5291	granite	5.3

ACA, Annapurna Conservation Area; SNP, Sagarmatha National Park

organic matter; that is, organic matter blown from lower elevations to higher elevations. However, recent studies of microbial communities at high elevations (Freeman et al. 2009, King et al. 2008, Schmidt et al. 2009) have caused us to reevaluate how life is sustained at these elevations. We have been studying microbial life in soils up to 6000 masl in the high Andes of South America and the southern Rocky Mountains of the United States for the past ten years. Although we have observed pockets of aeolian-supported life (Ley et al. 2004), we have found much larger areas of wind-swept lands that do not accumulate high amounts of organic debris from lower elevations but are nonetheless teeming with previously unreported microbial life (King et al. 2008, Schmidt et al. 2009). These studies have shown that in many subnival soils, microorganisms obtain their sustenance not from wind-blown organic matter but primarily from atmospheric gases through the processes of microbial photosynthesis and nitrogen fixation (Freeman et al. 2009, Schmidt et al. 2008, 2009) and that the buildup of microorganisms may be largely limited by soil water availability (King et al. 2008). Thus, although we have pushed our understanding of high-elevation life beyond the pioneering efforts of Swan (1963, 1990, 1992), the question remains as to whether these new discoveries made in the Andes and Rocky Mountains apply to even higher mountain ranges of the world.

The large area occupied by subnival soils in the Nepalese Himalayas (**Figure 1b**) makes it particularly important to understand the activity and abundance of high elevation microorganisms there. Here we examine microbial biomass and extracellular enzyme activity in subnival soils from the Annapurna and Sagarmatha (Everest) regions of the Nepalese Himalayas. Our study describes microbial life in four subnival soils: plant-covered, eroded previously vegetated, fine shale-derived gravel, and fine granite-derived gravel. This study is the first report of an ongoing research effort to characterize the microbial activity and diversity of the subnival soils of the Himalayas.

Methods

Study sites and sample collection Our sampling sites were in the Annapurna Conservation Area (ACA, Panthi et al. 2007, Shrestha et al. 2007) and the Sagarmatha National Park (SNP, Byers 2007) of the Himalayan Mountains in Nepal (**Figure 1, Table 1**). In each region, we sampled mineral soils from sites just above the highest plants and from sites as high as were attainable due to prevailing conditions (e.g. presence of ice and snow) at the time of sampling. Sampling was conducted in October and November of 2008 in order to take advantage of the seasonal lack of precipitation. Low precipitation seasons are ideal for this type of descriptive study because it minimizes short term variability due to individual precipitation events when comparing across sampling sites and allows access to the highest possible soils due to the relatively snow-free conditions. In addition to unvegetated soils of the ACA, we also sampled patches of soil dominated by the sedge *Kobresia* cf. *pygmaea* (C. B. Clarke) C. B. Clarke as well as soil from eroded areas adjacent to patches of *Kobresia*. These sites were located just below the lowest-elevation plant-free site. Soil samples were collected to a depth of 5 cm and placed in sterile zip-lock bags. Samples were frozen in the field by packing sample bags in a cooler along with snow and ice collected from the landscape and transported back to the laboratory over about a week. Samples were immediately extracted for K_2SO_4 dissolvable C and N (Weintraub et al., 2007) and then stored at –20°C for further analysis.

The low-elevation sites in the ACA are unvegetated, south-east facing slopes (28°43'N, 83°55'E) at 5122 masl and approximately 8 km east of the northern edge of Tilicho Lake. We also collected three samples from patches of *Kobresia* covered soil as well as three samples from eroded areas adjacent patches of *Kobresia* on a south-south-west facing slope (28°42'15"N; 83°56'05"E) at 4824 masl. The high-elevation sites in the ACA are unvegetated south-east facing slopes from 5503 to 5516 masl, 1 km north of Thorong Pass (28°48'N, 83°56'E). The Thorong Pass crosses the divide separating the Marsyangdi River to the east and the Kali Gandaki River to the west. All of the ACA sites are located on shale bedrock.

The sampling sites in the SNP receive significantly more precipitation than do the ACA sites (**Figure 1c**). We collected 4 samples from each of three unvegetated south-east facing slopes in this region, Chola Pass, Gokyo Lake 5, and Island Peak. All of the SNP sites were located on granite bedrock.

Soil temperature Soil temperature measurements were recorded using HOBO data loggers (Pendant temp/light, UA-002-64, Onset Computer Corp., Bourne, MA) from October 16th to 19th 2008 at the ACA *Kobresisa* dominated site. One data logger was placed flush with the soil surface and another at a depth of 4 cm.

Microbial biomass carbon and nitrogen Soil dissolved organic carbon (DOC), total dissolved nitrogen (TDN), and microbial biomass carbon (MBC) and nitrogen (MBN) were determined using the methods described in Weintraub et al. (2007). For soil DOC and TDN, 5 g of each soil sample was shaken with 25 ml of 0.5 M K_2SO_4 for 1 hour. For microbial biomass C and N, 5 g of soil was added to a 250 ml glass flask with 2 ml of chloroform, sealed and fumigated for 24 hours, and then vented for 1 hour; 25 ml of 0.5 M K_2SO_4 was added to each flask, and then they were shaken for 1 hour. Solutions were pre-filtered using a 1 μm Pall glass fiber filter (Pall Corporation, East Hills, NY). Solution C/N analysis was performed using a Shimadzu total organic carbon analyzer (TOC 5000) equipped with a total dissolved nitrogen (TDN) module (Shimadzu Scientific Instruments, Inc., Columbia, MD).

pH determination In order to determine soil pH, 2 g of each soil was placed to an individual 15 ml conical tube to which was added 2 mL of distilled water. Conical tubes were placed horizontally on a shaker table and shaken for 1 hr at 175 rpm. Soil pH was measured using a glass Fisher pH probe (Fisher Scientific, Pittsburgh, PA).

Extracellular enzyme activity Microbial extracellular enzyme activities were assayed using a modification by King et al. (2008) of the method of Weintraub et al. (2007). Enzymes assayed were: N-aceytalglucosaminase, cellulase (β-glucosidase), α-glucosidase, β-xylase, cellobiosidase, leucine aminopeptidase and organic phosphatase. For each sample, 2 g of soil was added to 150 ml of buffer at the average pH for the soil type (0.5M Acetate at pH 5 for granitic, 0.5M Acetate at pH 6 for eroded and vegetated, and 0.5M Bicarbonate at pH 7.3 for shale derived soils) and homogenized at 3000 rpm for 1 minute using a Ultra-Turrax homogenizer (IKA Works Inc., USA). Soil slurries were assayed using the same controls, fluorescent substrates, and solution volumes as in King et al. (2008). Soils were incubated with substrates for 20 hours at 14°C.

Water holding capacity Tubes for assaying water holding capacity (WHC) were constructed by cutting the bottom off of a 1 cm diameter 15 ml conical tube and covering the opening with 1-mm gauge plastic mesh. The mesh was wetted with deionized water prior to the addition of soil to the tube so that particles less than 1 mm in size would clump together at the bottom of the tube. For each sample we added ~4 g of soil to a tube and then added ~2 ml of H_2O. Wetted sample tubes were placed in 50 ml conical tubes, which were drained periodically. When an individual sample stopped dripping, the mass of the sample was recorded. Samples were then dried at 100ºC for 24 hours. Water holding capacity is reported as the g H_2O at soil saturation divided by g dry soil.

Statistics and data analysis Tukey's honestly significant difference tests (Devore 2004) were performed in R (version 2.8.1, 12/22/2008, R Foundation for Statistical Computing http://www.r-project.org/index.html). A correlation test (Devore 2004) between microbial biomass C (MBC) and water holding capacity was also performed in R.

Results

Microbial biomass and extracellular enzyme activity were extremely low in the shale-derived soils from the ACA (**Figure 3, 4**). The granitic soils of the SNP had significantly higher biomass and activity than the shale-derived soils (**Figure 3, 4**). As expected, soils of the lower-elevation eroded area had higher microbial biomass than either of the two higher-

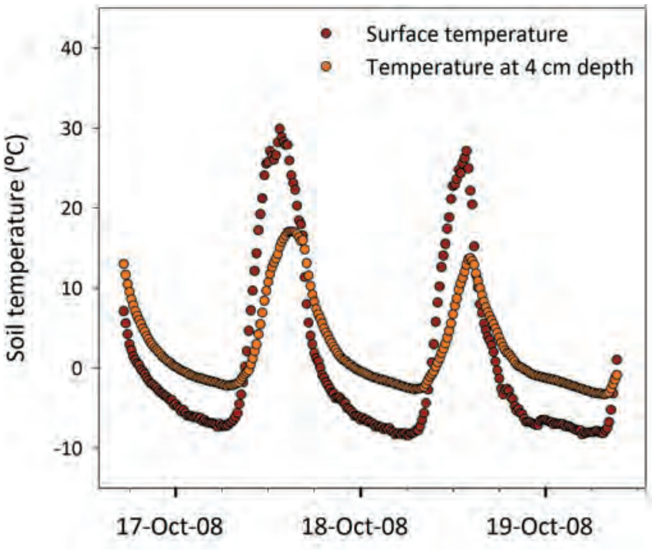

Figure 2. Soil temperature measurements from two days near the 4824 m elevation *Kobresia* dominated site in the ACA region.

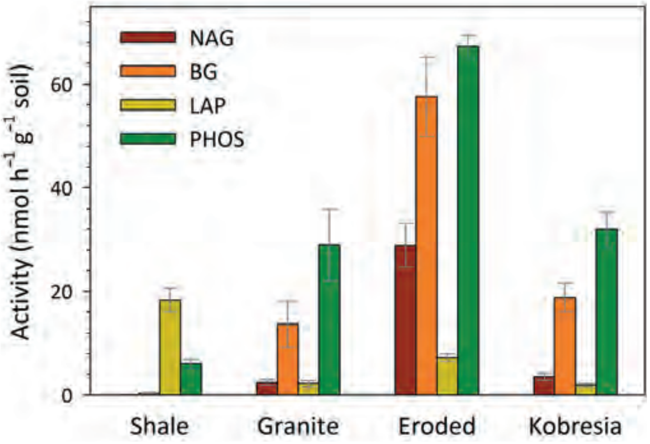

Figure 3. Extracellular enzyme activities for the four soil types. Enzymes activities shown are N-aceytalglucosaminase (NAG), cellulase (β-glucosidase) (BG), organic phosphatase (PHOS) and leucine amino peptidase (LAP). α-glucosidase and β-xylase activities were at similar levels to NAG and are not shown. Significant differences are designated by letters grouping similar levels of activity for an individual enzyme (Tukey's Test, $p < 0.05$). Error bars are standard error.

elevation mineral soils, and the *Kobresia* soils had the highest microbial biomass C and N (**Figure 4**). Surprisingly, while both the eroded soils and the *Kobresia* soils had higher enzyme activity than the mineral soils, the eroded soils had significantly higher extracellular enzyme activity than the vegetated soils (**Figure 4**). The biomass trends were mirrored by the water holding capacity measurements (**Figure 5**). Finally, there was a significant correlation between MBC and WHC for all samples (**Figure 6**, Pearson $r^2 = 0.426$, correlation test: $p < 0.001$). However, this trend was primarily driven by the increase of WHC and MBC with plant colonization.

Discussion

The subnival soils examined in this study are subject to some of the most extreme environmental conditions of any soils on Earth. Nevertheless, we found measurable amounts of microbial biomass and enzyme activity in even the most visually barren mineral soils (**Figure 3, 4**). Perhaps due to such extreme conditions, the biomass C numbers we observed were on average very low for both the mineral and the vegetated soils we sampled. Particularly low microbial biomass was found in the shale-derived mineral soils of the ACA. At an average of 20 µg C/g soil, these biomass levels

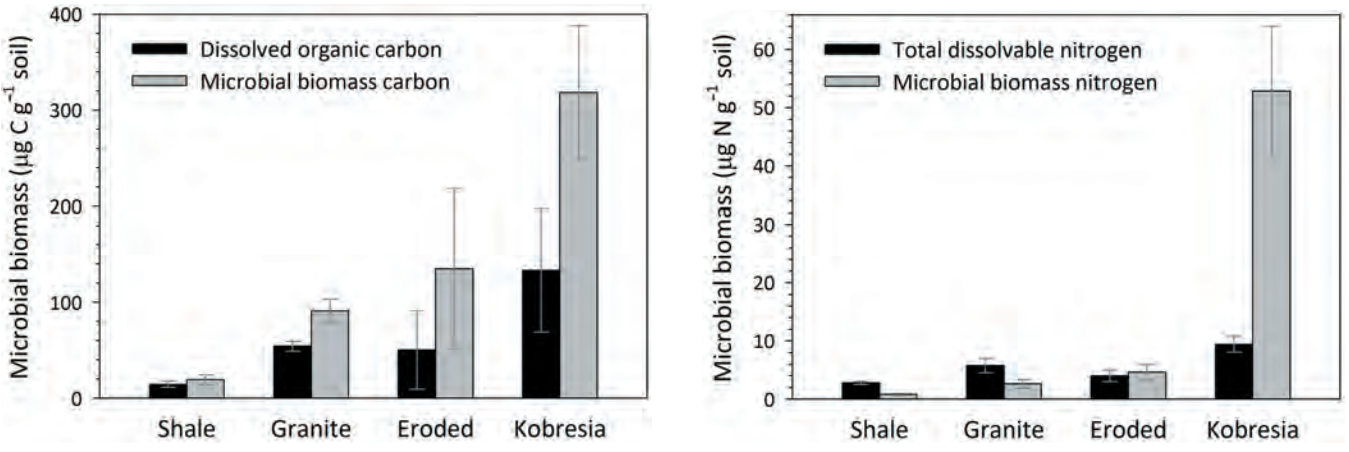

Figure 4. (a) Dissolved organic carbon (DOC) and microbial biomass carbon (MBC) for the four soil types. (b) Total dissolvable nitrogen (TDN) and microbial biomass nitrogen (MBN) for the four soil types. Significant differences are designated by letters grouping similar levels (Tukey's Test, $p < 0.05$). Error bars are standard error.

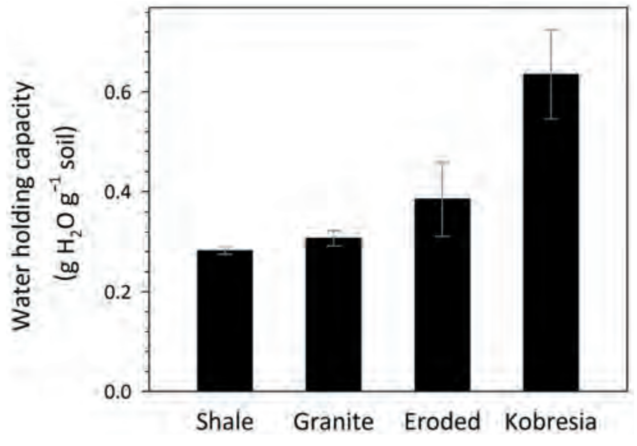

Figure 5. Water holding capacity (g H_2O/g dry wt of soil) for the four soil types. Significant differences are designated by letters grouping similar levels of activity for an individual enzyme (Tukey's Test, $p < 0.05$). Error bars are standard error.

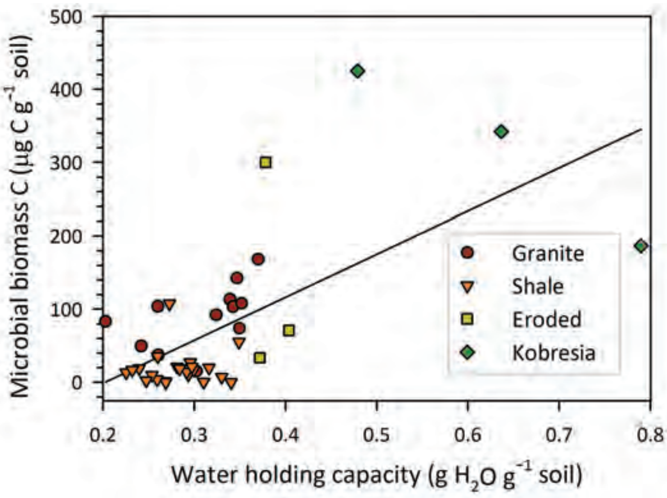

Figure 6. Microbial biomass C versus water holding capacity for all samples (Pearson $r^2 = 0.426$, correlation test: $p < 0.001$). It is apparent that this trend is driven by the increase in WHC and MBC with plant colonization. Taken individually the only sample group with a significant positive association between WHC and MBC was the granitic soils ($r^2=0.301$, $p<0.05$). Error bars are standard error.

are the lowest reported to date for subnival or recently deglaciated soils. Previously, the lowest microbial biomass numbers were reportedly found in alpine and Antarctic glacial moraines, which harbor 60 and 100 µg C/g soil, respectively (Tscherko et al. 2003a, b). Likewise, subnival-zone soils of Colorado and Peru contained 80 and 140 µg C/g soil, respectively (King et al. 2008). Thus, subnival soils may represent environments at the upper boundary of suitable conditions for sustaining microbial life. More work is needed to determine what component of the biomass is active at these sites and what component consists of dormant organisms blown in from lower elevations.

Our results support the hypothesis put forth by King et al. (2008) that the primary limiting factor determining microbial biomass levels in these extreme subnival soils is water availability (**Figure 6**). Indeed, the shale-derived plant-free soils from the ACA had the lowest water contents of any of the soils and the lowest measured levels of microbial biomass. These soils also had the lowest water holding capacities of any of the soils examined in this study (**Figure 5**). However, neither soil water content nor water holding capacity was significantly different among subnival soil sampling areas. Interestingly, the granitic soils of the SNP had similar microbial biomass levels to the granitic soils of the Rocky Mountains of the central United States that we have previously studied (King et al. 2008). In that same study we reported that shale-derived plant-free soils from the Andes in Perú had higher microbial biomass than the Rocky Mountain soils, however, the Himalayan soils display the reverse trend. This discrepancy suggests that rock type is not the main factor determining microbial biomass levels. Indeed, this trend is mirrored by the lower extracellular enzyme activities and DOC content of the shale-derived soils versus the granite-derived soils from the Nepalese Himalayas. These results point to possible differences in soil age and development between the barren subnival soils of the ACA and the SNP, differences that may originate from variation in slope stability due to degree of slope, bedrock hardness, or annual precipitation (Gabet 2004). As seen in **Figure 1c**, the SNP area receives greater precipitation than does the ACA, perhaps resulting in higher rates of weathering and soil formation in the SNP. However, once soil succession proceeds to the point of plant colonization, significant accumulation of microbial biomass does occur. Thus, while water holding capacity may only be a fair predictor of microbial biomass it appears to be a reasonable proxy for subnival soil development.

Although there is not a significant influence of soil bedrock on the overall microbial community biomass, we can see that the pH of the soils varies significantly depending on bedrock type and degree of plant colonization. Fierer and Jackson (2006) have shown that microbial community composition can vary significantly with changes in soil pH and Sinsabaugh et al. (2008) have demonstrated that this pH variation can result in different rates of extracellular enzyme activity. Indeed we see that cellulase (β-glucosidase) activity is very low in the shale barren soils (high pH) while it is one of the predominant enzymes in the granitic barren soils (low pH). The opposite trend was seen with protease (leucine aminopeptidase) activity, wherein activity was high in the barren shale soils and barely detectable in the granitic soils. Furthermore, as the soils became more *Kobreisia*-dominated there was an increase in extracellular enzyme activity in concert with a shift in soil pH. Therefore, as soils development proceeds, the differences in nutrient cycling between barren soil types may disappear. Moreover, the shift in patterns of snow accumulation and overall precipitation predicted to occur as the climate warms (Beniston 2003) may significantly influence rates of soil weathering and water holding capacity. Ultimately, said increases in soil weathering may alter the differences between bed rock types and result in an overall increase in productivity, biomass, and activity of these high altitude soils.

The microbial biomass C levels in the *Kobresia*-dominated soil patches of the ACA, with an average of 325 µg C/g soil, were also low for an alpine dry meadow community and suggestive of a low productivity system; *Kobresia myosuroides* dominated soils from the Rocky Mountains of Colorado, USA average 1260 µg C/g soil (King et al. unpublished data). Once again, the low precipitation of the ACA region may be the cause of the low microbial biomass by directly limiting plant photosynthesis and indirectly limiting the amount of carbon available to soil heterotrophs. However, relative to the soil C levels, the Nepal *Kobresia* soils had very high microbial N content (~54 µg N/g soil); levels very similar to *Kobresia* soils in Colorado (75 µg N/g soil, Fisk et al. 1998). This relationship results in a lower microbial C:N ratio for the Nepal sites (6.0) versus Colorado (16.8), further indicating increased microbial C-limitation for the Nepal soils.

High microbial N levels late in the fall may also be an adaptation that fosters N retention in this ecosystem over the winter. A similar retention mechanism occurs in other ecosystems via microbial growth on senescing plant material after the plant growing season, resulting in immobilization of N especially in seasonally cold (Jaeger et al. 1999, Zak et al. 1990) or seasonally dry (Singh et al. 1989, Vitousek and Matson, 1984) systems. This explanation is further supported by the low extractable nitrogen levels of the bulk soils from the *Kobresia* soil patches of the ACA (10 µg/g soil). Regardless of the mechanism for N storage, the data suggest that the *Kobresia* soil communities of the ACA have high N retention in the face of low productivity.

A final surprising result of our study is the finding that although *Kobresia* sward soils had the highest biomass of subnival Nepalese soils, the eroded soils adjacent to the *Kobresia* soils had the highest enzyme activity. This is likely a result of the eroded soils losing their structure, allowing the microbial community greater access to sequestered organic matter. This effect would be compounded by cessation of rhizodeposition of labile carbon sources, which could cause the microbial community to shift towards breaking down more recalcitrant organic matter, thus the increase in enzyme activity. In addition, the lower nitrogen availability in the eroded soils relative to the *Kobresia* sward may be responsible for the concurrent stimulation of N scavenging enzymes such as NAG and LAP. These effects have been theorized to explain the shift in dominance of extracellular enzyme production in nutrient limited environments (Wallenstein and Weintraub 2008), but the majority of evidence for this phenomenon comes from studies of microorganisms in culture (Harder and Dijkhuizen 1983). Our results in the eroded soils support the long held hypothesis that high microbial enzyme activity should be higher in soils that have low amounts of labile nutrients relative to complex organic substrates.

Conclusions

The levels of microbial biomass and activity we observed in the subnival zone soils of the ACA and SNP regions of the Nepalese Himalayas are some of the lowest ever observed for this, or any, ecosystem type. It is likely that the subnival zone will further grow in size as a result of high-elevation glacial melt and in some areas by overuse of alpine grazing ranges, significantly increasing the already large area of Nepal that is occupied by this ecosystem type. The evidence from our work suggests that expanding areas of the subnival zone will be characterized by low levels of microbial biomass that increase gradually with water holding capacity and soil development. However, specific activity levels will be dependent on nutrient availability, soil pH, speed of soil development, and regional precipitation patterns. Our preliminary survey of microbial biomass and activity in the subnival zone of the Nepalese Himalayas reinforces the notion that the organisms that live in this precarious ecosystem are subjected to some of the most extreme environmental conditions on Earth. Further study of these areas has the potential to uncover novel microbial communities in these fascinating soils from the highest mountain range on Earth.

Acknowledgements

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Regeneration of *Pinus wallichiana* AB Jackson in a trans-Himalayan dry valley of north-central Nepal

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We studied the elevational pattern of forest composition and regeneration of the subalpine conifer tree species *Pinus wallichiana* in Manang, a trans-Himalayan dry valley in north-central Nepal. Thirty-five quadrats (10 m × 10 m) were laid between 3300 and 4000 masl on both north- and south-facing slopes. We measured diameter at breast height (DBH) of each mature individual of all tree species (DBH ≥10 cm), and recorded the number of seedlings (DBH <10 cm, height <30 cm) and saplings (DBH <10 cm, height >30 cm). We also measured soil moisture and soil pH, estimated canopy cover, and recorded slope and altitude in each quadrat. For all species together and for several species individually, tree density, seedling density, sapling density and tree basal area were found to decrease with elevation on both north and south aspects. This trend is largely explained by the progressively harsher environment at higher elevations. The north-facing slopes in our study area have denser forests than the south-facing slopes, the density of all size classes (seedling, sapling and mature plants) and basal area being greater on the northern aspects. These aspect-wide differences are attributable to the stark difference in soil moisture between northern and southern aspects, which is in turn due to the difference in insolation. Irrespective of elevation and aspect, all the forests studied are regenerating, as indicated by inverse J-shaped density-diameter curves. The elevational pattern of seedling and sapling abundance is explained only by elevation. Whereas other variables (*e.g.*, canopy) are considered to have an important influence on seed germination and seedling establishment, they turn out not to be significant predictors of density of seedlings and saplings. This failure to identify a relationship is probably due to our use of non-parametric test (tree regression analysis) that we used to establish the relationship between density and its potential explanatory variables or due to our selection of 1 standard error rule yielding sub-optimal models for regression trees.

Key words: density-diameter curve, regeneration, seedling, sapling, altitude, canopy, Manang Valley

Pinus wallichiana AB Jackson (Himalayan Blue Pine) is native to the Himalaya, Karakoram, and Hindu Kush mountains. It has an extensive distribution and grows all along the Himalayas in an almost continuous range from eastern Afghanistan and Pakistan through India, Nepal, Bhutan, Myanmar, and China at elevations between 1800 and 4300 masl (Critchfield and Little 1966, Polunin and Stainton 1997). The plant is generally found in valleys and foothills, sometimes in pure stands but often in association with other conifers including *Cedrus deodara*, *Abies pindrow*, *Picea smithiana* and *Juniperus indica* [*Juniperus excelsa* subsp. *polycarpos*], and with broadleaved species including *Quercus semecarpifolia*, *Betula utilis*, *Acer* and *Ilex* species (Earle 2009). *Pinus wallichiana* forests grow in drier areas susceptible to fire where the plant grows as an early successional species (Numata 1966, Stainton 1972, Ohsawa et al. 1986). In wet areas, the plant grows in secondary forests (Polunin and Stainton 1997). *P. wallichiana* is an important source of timber and fuel for villagers in mountain valleys and is also important in protecting the upper parts of mountain watersheds (Ives and Messerli 1989, Stainton 1972).

Regeneration of tree stands depends on a combination of factors controlling seed availability, germination, seedling growth and establishment (Greene et al. 1999, Dovciak et al. 2003). Whereas environmental conditions play an important role in establishment and distribution of seedlings (Bonnet et al. 2005), regeneration of dominant trees in dry valleys is influenced even by small-scale human impacts. Under such impacts, the typical inverse J-shaped DBH (diameter at breast height) class distribution observed among forest species, where frequency of individuals in larger size classes falls systematically and progressively, resulting in a non-linear relationship between frequency and size class, generally gives

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Table 1. Description of study area

Aspect	Transect	Locality	Elevation (masl)	Dominant tree species	Number of quadrats sampled
North	1	Above Humde	3300–3500	*Pinus wallichiana* *Juniperus indica*	10
		Above Humde	3500–3800	*Pinus wallichiana* *Abies spectabilis*	15
	2	Above Gangapurna lake	3800–4000	*Betula utilis* *Pinus wallichiana*	10
South	3	Ngawal side	3300–3500	*Pinus wallichiana* *Juniperus indica*	10
		Ngawal side	3500–3800	*Juniperus indica*	15
	4	Above Braga	3800–4000	*Juniperus indica*	10

way to a sporadic and/or unimodal distribution (Wangda and Ohsawa 2006a). Inverse J-shaped distribution is indicative of a forest in a state of regeneration (Kimmins 1987). A shift from inverse J-shape to unimodal or multiple-peaked distribution is the result of substantial changes in the state and pattern of forest regeneration, suggesting that the forest is in trouble. Such a shift is generally caused by both anthropogenic and natural causes (climatic or biotic), as reported for *P. roxberghii* forest on dry valley slopes along the Punatsangchu river in west-central Bhutan (Wangda and Ohsawa 2006a). Several studies have focused on the altitudinal and latitudinal forest zones of the Himalayas in relation to climate (Saxena and Singh 1984, Ohsawa 1987, Tang and Ohsawa 1997, Wangda and Ohsawa 2006a). However, little is known about the comparative ecology and regeneration dynamics of the forest along the typical dry valley slopes, which are particularly important because the steep environmental gradients are associated with rapid change in forest types (Wangda and Ohsawa 2006b).

Regeneration and seedling distribution in conifer forests have been shown to be influenced by both large-scale disturbances such as wildfire and forest clearing (Turner et al. 1998, Bonnet et al. 2005) and also small-scale disturbances such as animal grazing (wild and domestic), lightening and disease (Bonnet et al. 2005). Most studies on regeneration of *Pinus* species have focused on such disturbances (e.g., Spanos 1994, Spanos and Spanos 1996, Spanos et al. 2000, Scott et al. 2000, Spanos et al. 2001, Bonnet et al. 2005, Darabant et al. 2007). A few studies investigated the effect of physical factors, such as aspect and elevation (Wangda and Ohsawa 2006a, Mong and Vetaas 2006), on forest regeneration. In this study, we examine forest composition and regeneration patterns of *P. wallichiana* along an altitudinal gradient on slopes of the dry Manang Valley in north-central Nepal, with particular attention to the differentiation of slopes with northerly and southerly exposure. Principle objectives of this research were (a) to determine the community structure of *P. wallichiana* forest, (b) to understand the regeneration pattern of *P. wallichiana*, and (c) to identify environmental factors that affect regeneration of *P. wallichiana*.

Materials and methods

Study area The study area lies in Manang Valley (also called Nyeshang) of Manang District in north-central Nepal. This trans-Himalayan Valley (28° 37' – 28° 39' N, 83° 59' – 84° 08' E) is located in the northern part of the Annapurna Conservation Area. The valley is traversed by the Marsyangdi River and is surrounded by the high peaks (>6000 masl) of the Annapurna range to the south, Manasalu to the east, Peri, Himlung and Choya to the north, and Damodar and Muktinath to the west. The climate of the valley is dry, characteristic of the trans-Himalayan region. It is a rain shadow area with a mean annual precipitation of 444 mm (at 3420 masl) and mean annual temperature of 6.2°C (Miehe et al. 2001). The area is covered by snow from November to March. Snow melt water is the main source of soil moisture in forested areas (Shrestha et al. 2007). The south-facing slope (south aspect) is substantially drier than the north-facing slope (north aspect) (Bhattarai et al. 2004). Vegetation composition on the northerly and southerly aspects is quite distinct. *P. wallichiana, Abies spectabilis, Betula utilis, Juniperus indica* and *Salix* species are the common tree species on north facing slopes, while on the southern slope *B. utilis* and *A. spectabilis* are found only in moist gullies. The natural forest in the valley extends from 3000 to 4200 masl on the north aspect, while its upper limit is below 4000 masl on the south aspect (Panthi et al. 2007).

Our sampling was carried out along four vertical transects, two in each aspect. Because a single transect never spanned the elevational limits of our study area (3300–4000 masl), the entire range was represented by transects at two sites with similar physical environment and biotic composition. On the northern aspect, the two transects were laid at Humde village (3300–3800 masl, 28° 37' 39" N, 84° 05' 30" E) and near Gangapurna Glacier Lake (3800–4000 masl, 28° 39' 42" N, 83° 59' 44" E) (**Table 1**). South-aspect transects were laid near Ngawal village (3300–3800 masl, 28° 38' 23" N, 84° 05' 22" E) and above Braga village (3800–4000 masl, 28° 39' 54" N, 84° 03' 24" E – on the way to Ice Lake). The average slopes of the sampling sites on the northern and southern aspects were 22° and 17°, respectively. The forest on the northern aspect of the valley had been damaged severely by forest fire about 35 years prior to our study (Shrestha et

Table 2. Density (individuals/ha), basal area (BA, m^2/ha) and Importance Value Index (IVI, %) of tree species (excluding seedling and sapling) at various elevation bands

	Northern Aspect				Southern Aspect			
Elevation (masl)	**Tree species***	**Density (per ha)**	**BA (m^2/ha)**	**IVI (%)**	**Tree species***	**Density (per ha)**	**BA (m^2/ha)**	**IVI (%)**
3300–3500	PW	1028	18.6	75.63	PW	570	11.8	61.64
	JI	200	0.15	11.86	JI	320	5.015	38.35
	BU	50	0.06	3	BU	0	NA	NA
	S	50	0.02	4.27	S	0	NA	NA
	AS	100	0.21	5.33	AS	0	NA	NA
	Total	**1428**	**19.04**	**100.09**	**Total**	**890**	**16.815**	**99.99**
3500–3800	PW	190	2.76	28.09	PW	59	0.42	34.01
	JI	100	0.037	19.44	JI	517	0.22	60.95
	BU	267	3.44	9.64	BU	0	NA	NA
	S	33	0.02	3.98	S	17	0.017	5.08
	AS	137	1.02	38.9	AS	0	NA	NA
	Total	**727**	**7.277**	**100.05**	**Total**	**593**	**0.674**	**100.04**
3800–4000	PW	240	2.26	39.16	PW	0	NA	NA
	JI	0	NA	NA	JI	375	NA	NA
	BU	225	1.46	41.82	BU	0	NA	NA
	S	0	NA	NA	S	0	NA	NA
	AS	150	0.36	19	AS	0	NA	NA
	Total	**615**	**4.08**	**100.08**	**Total**	**375**	**-**	**-**

* PW = *Pinus wallichiana*; JI = *Juniperus indica*; AS = *Abies spectabilis*; BU = *Betula utilis*; S = *Salix* species

al. 2007); we observed a large number of burnt logs. Because *P. wallichinana* is used as a construction material, there were many stumps, left behind after logs had been extracted.

Field sampling For the sake of analysis, we divided the elevational range into three bands: 3300–3500, 3500–3800 and 3800–4000 masl. These elevation bands are different in physical environment (both temperature and moisture) as well as in biotic composition (Mittermeier et al. 2004, Chaudhary 1998). For sampling, 35 square quadrats (10 m × 10 m) were randomly placed on each aspect such that five of them always occupied a 100 m elevation band. In each quadrat, we recorded the number and diameter at breast height (measured at 1.37 m above the ground surface) of individual trees (DBH ≥ 10 cm) of each species. Canopy cover was estimated visually. We divided each quadrat into four sub-quadrats (5 m × 5 m), and recorded the number of saplings (DBH <10 cm, height >30 cm) and seedlings (DBH <10 cm, height <30 cm, Rao et al. 1990) of each tree species in two diagonally located sub-quadrats, selected at random. Moisture and pH of soil were measured at the four corners and center of each quadrat with the use of a soil pH and moisture tester (Model DM 15, Takemura Electric Works Ltd., Japan); the values were averaged for data analysis.

Numerical methods and statistical analysis Density (individuals/ha), frequency (%), basal area (m^2/ha), and the importance value index (IVI) (Holdridge et al. 1971) of trees were calculated from field data. We also determined the density of seedlings and saplings of tree species. To understand the regeneration status of *P. wallichiana,* trees in the three elevation bands were divided into 10 cm interval size classes based on DBH.

How do the physical and biotic differences between the north and south aspects affect seedling and sapling abundance? We compared the densities between the two aspects. Because the assumptions of parametric statistical tests (in this case, independent sample t-test) were violated (by non-normality in distribution of cases and heterogeneity of variances of groups being compared), we performed the non-parametric Wilcoxon–Mann–Whitney two-sample rank-sum test in SPSS 16.0 (SPSS Inc.).

Finally, we attempted to establish what determines density of seedling and sapling. We measured a range of environmental variables on the studied plots: soil pH, soil moisture, canopy cover, slope and altitude. Because these explanatory variables can correlate among themselves – which is always the case in field-based ecological studies – we tried to determine the significant predictors of density using multiple regression. However, the residuals in regression analysis were severely non-normal, and we observed both linear and non-linear relationships between explanatory and dependent variables. This indicated the violation of one of the assumptions of regression analysis and the likelihood that power transformation would not be able to fix normality. Because it entails fewer assumptions, tree regression is a useful alternative to multiple regression analysis. We therefore performed tree regression analysis of our data in order to relate explanatory variables to the dependent variables in R 2.9.1 (The R Foundation for Scientific Computation).

One approach to building the best model (simplest model that best fits the data) is to begin with the simplest model and make it increasingly complex until the model keeps on improving in its fit to the data. This would entail growing the tree with more and more splits. As Breiman and colleagues have pointed out problems in this approach in their seminal work (Breiman et al. 1984), we first allowed the tree to grow to the maximum (full) size and then pruned it so that only important predictors remain in the simplified model (Rejwan 1999, De'ath 2000). The minimum cross-validated error rate was determined for tree models of various sizes. The smallest tree with an error rate within 1 SE of the minimum error rate was chosen as the best model (Breiman et al. 1984) after 1000 simulations.

All environmental variables (soil pH, soil moisture, canopy cover, slope and altitude) were accommodated in the model although cross-validation would eventually select only the important ones. On top of these, we used density of sapling as a potentially explanatory variable for explaining seedling abundance because the germination of seed and their growth to seedling is likely to be affected by nearby saplings which are bigger in size and therefore more effective competitors. For the same reason, we did not use seedling density as a potentially explanatory variable for explaining sapling abundance.

Results

Forest structure Total tree density and basal area decreased from low to high elevation on both aspects (**Table 2**). This pattern was generally followed by *P. wallichiana*. However, some species, e.g., *B. utilis* and *A. spectabilis,* had their lowest tree density and basal area values in the lowest elevation band. Because *P. wallichiana* is the most dominant species in these forests, its elevational pattern of density and basal area determined that of the forest as a whole. Forest density was greater on the northern aspect than on the southern. Above 3700 masl on the southern aspect no tree individuals of *P. wallichiana* were observed, and *J. indica* was represented only by bushes or scrub with minimal effect on basal area. The treeline occurred at a lower elevation on the southern aspect (3800 masl) than on the northern aspect (4100 masl).

Northern aspects were occupied by seedlings and saplings much more densely than southern aspects (**Figure 1**). Using the non-parametric Wilcoxon–Mann–Whitney two-sample rank-sum test, we confirmed that the north-south differences are highly significant.

Regeneration of Pinus wallichiana The density-diameter curve of tree populations of *P. wallichiana* in all elevation bands resembled an inverse J-shape on both aspects (**Figure 2**). We observed larger trees on north

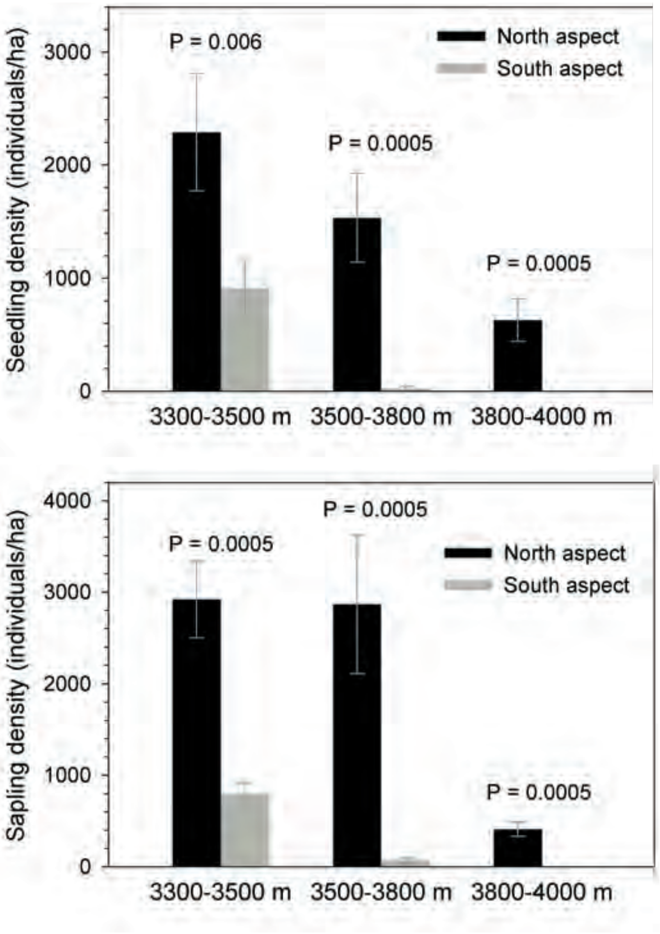

Figure 1. Seedling (top) and sapling (bottom) densities (mean ± 1 standard error). All the aspect-wise differences in the densities as determined by Wilcoxon–Mann–Whitney two-sample rank-sum test (two-tailed) are statistically highly significant.
Seedling density: Mann–Whitney $U = 14$, $n_1 = n_2 = 10$, $P = 0.006$ for 3300–3500m, Mann–Whitney $U = 15$, $n_1 = 15$, $n_2 = 14$, $P = 0.0005$ for 3500–3800 m, Mann–Whitney $U = 11$, $n_1 = 10$, $n_2 = 11$, $P = 0.0005$ for 3800–4000 m.
Sapling density: Mann–Whitney $U = 1.5$, $n_1 = n_2 = 10$, $P = 0.0005$ for 3300–3500 m, Mann–Whitney $U = 16.5$, $n_1 = 15$, $n_2 = 14$, $P = 0.0005$ for 3500–3800 m, Mann–Whitney $U = 0$, $n_1 = 10$, $n_2 = 11$, $P = 0.0005$ for 3800–4000 m.

aspect than on the south aspect (not shown in figure), and on valley floor and lower elevations than at higher elevations of both aspects (observation, **Figure 1**). Altogether 18% of plots on north and 40% of plots on south were devoid of mature trees.

Among the six variables, five environmental (soil pH, soil moisture, canopy cover, slope and altitude) and one biotic (density of saplings as a potential predictor for seedling density), the only significant predictor of both seedling and sapling abundance was altitude (**Figure 3**). On both aspects, seedling and sapling density decreased with altitude. Altitude explained approximately half of the total variance

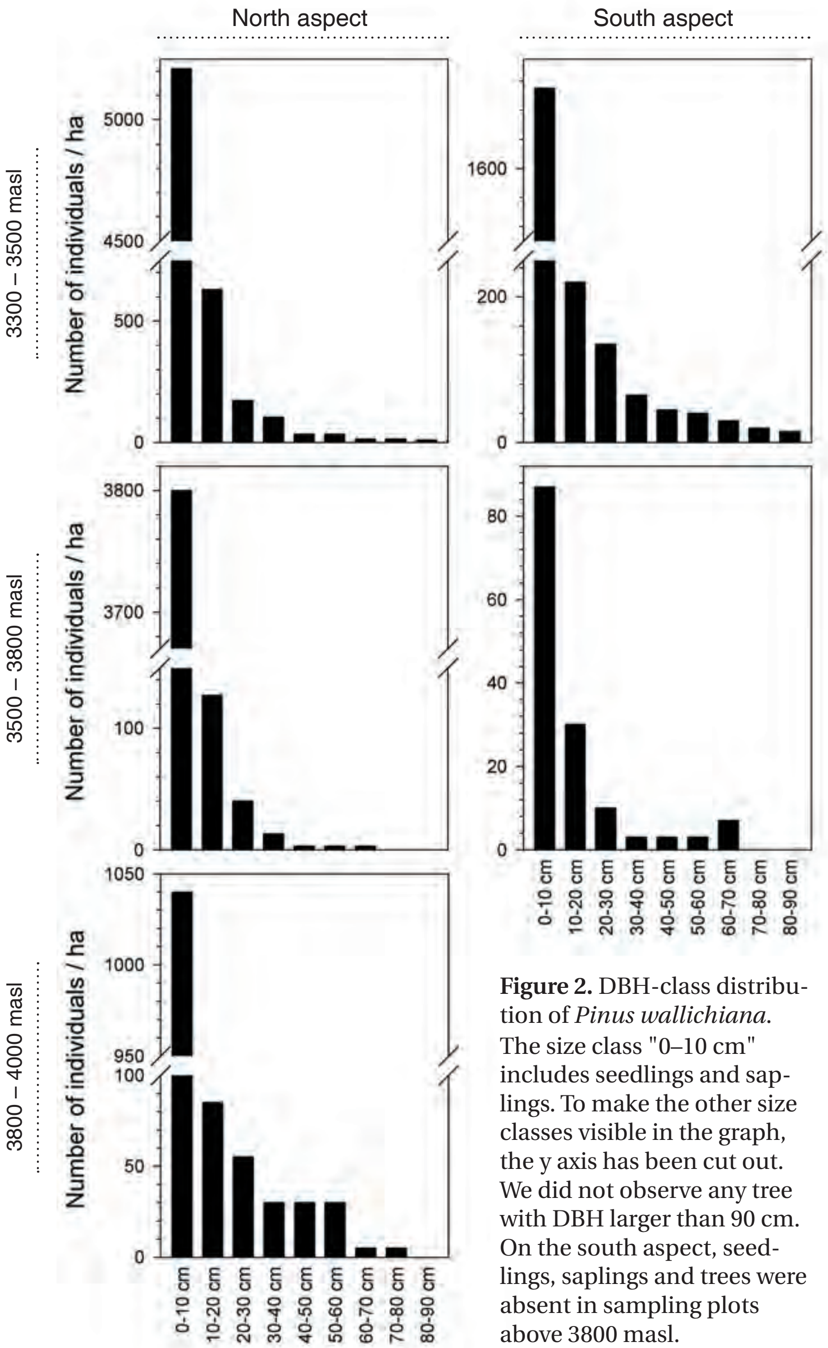

Figure 2. DBH-class distribution of *Pinus wallichiana.* The size class "0–10 cm" includes seedlings and saplings. To make the other size classes visible in the graph, the y axis has been cut out. We did not observe any tree with DBH larger than 90 cm. On the south aspect, seedlings, saplings and trees were absent in sampling plots above 3800 masl.

in abundance of seedlings and saplings in all cases except that it explained three-quarters of the total variance in sapling abundance on the southern aspect.

Discussion

Our transects were laid in the upper distributional ranges of the tree species. Towards the upper limit, environmental conditions become increasingly severe, resulting in the formation of a boundary of tree species called treeline. Various studies have repeatedly shown that some aspect of temperature – means or extremes – determine the position of treeline (Körner 1998, Jobbagy and Jackson 2000, Körner and Paulsen 2004). Studies have shown that annual increments in tree ring correlate with temperature at high elevation boundaries; for example, ring width of *Picea abies* and *P. cembra* growing near the alpine timberline in Switzerland positively correlates with summer temperature (Meyer 2000). Given the sharp decline in temperature with elevation – temperature lapse rate for the western Himalayas being 0.6–0.74°C/100 m for various months of the year (Jain et al. 2008) – and high sensitivity of physiological processes to temperature, it is not surprising that tree density, seedling density, sapling density and tree basal area decreased with elevation on both north and south aspects. High elevations are also characterized by shorter growing season, resulting in reduced annual growth (Tranquillini 1979, Vetaas 2000). This pattern of decrease in density and basal area with elevation, however, can vary with species because biotic interactions, importantly competition, also play a role in growth rate.

Northern aspects have denser forest than the southern aspects, with greater basal area and higher density of all size classes (seedling, sapling and mature plants) (**Figure 1, Table 2**). Five species of trees were found on northern aspects, but only three species on southern (**Table 2**). Higher species richness on northern aspects has been reported by previous studies (e.g., Panthi et al. 2007). The ecological significance of aspect is important because it influences diameter growth of tree, forest productivity, and species distribution (Hutchins et al. 1976, Verbyla and Fisher 1989). *B. utilis* and *A. spectabilis*, which are absent on the southern aspects, contributed substantially to the higher total tree density and, especially, total basal area found on the northern aspect.

The density-diameter curve of *P. wallichiana* populations resembled an inverse J-shape (**Figure 2**), indicating sustainable regeneration (Kimmins 1987, Shimano 2000). Shrestha et al. (2007) and Ghimire and Lekhak (2007) found inverse J-shape size-class distribution for *B. utilis* and *A. spectabilis,* respectively, in nearby forests on the northern aspect. Tree regression analyses show that altitude is the only significant predictor of seedling and sapling density on both aspects. Altitude explained half to three-quarters of the total variance in seedling and sapling density. Whereas we should not be surprised that altitude is the most important predictor of the seedling and sapling abundance, other variables (including canopy and aspect) that have been considered important

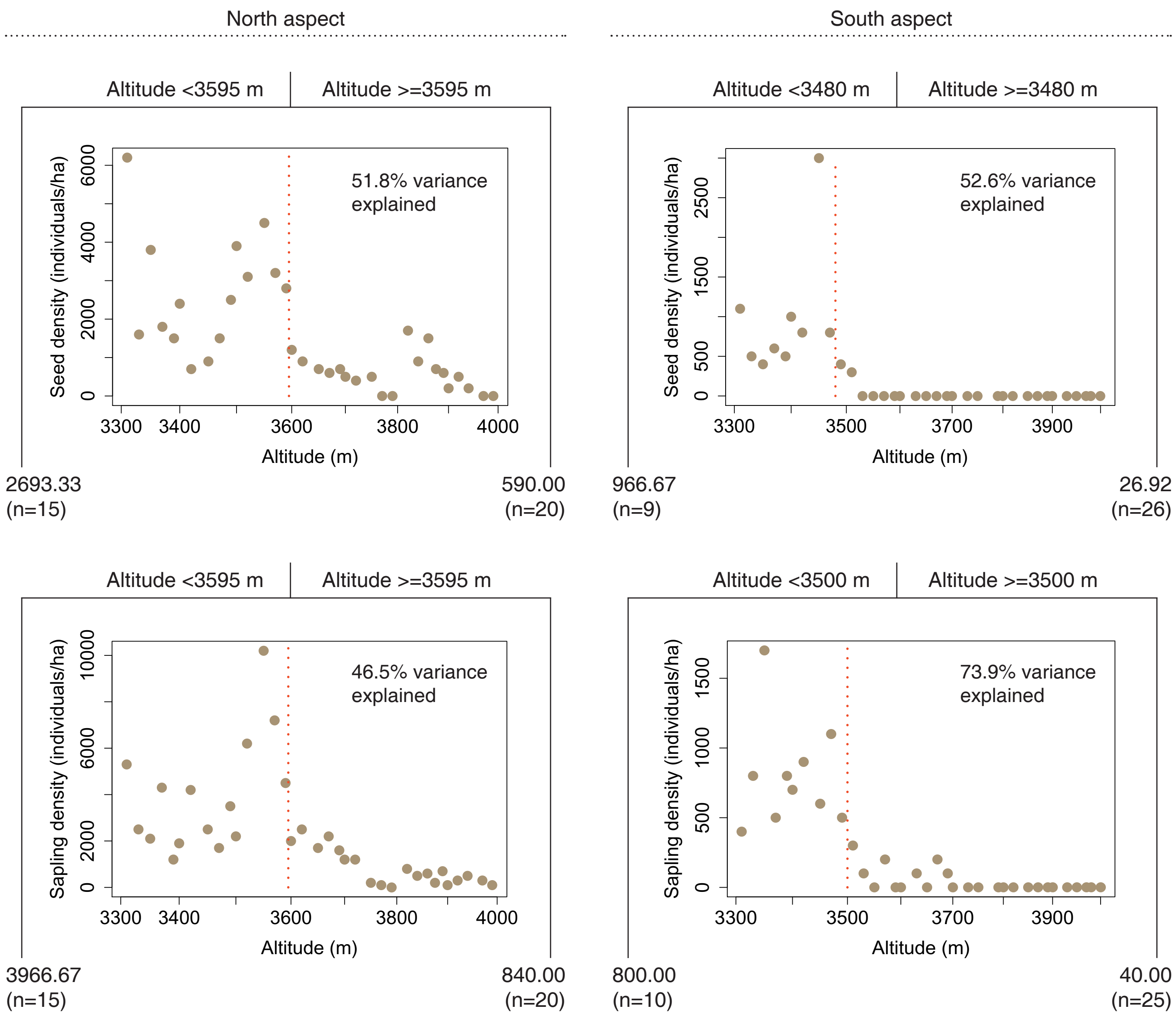

Figure 3. Regression trees relating abundance of seedlings and saplings to six potentially explanatory variables (soil pH, soil moisture, canopy cover, slope, altitude, and sapling density). In all cases the only significant explanatory variable was altitude, explaining approximately half of the total sum of squares except in one instance in which it explained 74% of the variance in sapling density. The tip of the tree branches list the mean of the response variable (density) assigned to the tree-branch and the sample size in parentheses.

in determining environmental conditions suitable for germination and growth of young plants do not turn out to be significant predictors.

Seedlings are generally light-demanding; they often require direct solar radiation (Tilman 1985). High canopy cover by large trees reduces the amount of direct sunlight that reaches floor; high canopy cover may also cause litter accumulation, which is not a favorable condition for seed germination and seedling establishment for *Pinus* (Neyisci 1993, Spanos et al. 2001, Bonnet et al. 2005). However, we failed to establish a relationship between canopy and density of seedlings and saplings. This finding may possibly reflect the actual absence of such a relationship in our study systems; we might have observed a correlation between density and canopy (r = 0.44 for both seedling and sapling, Pearson Correlation, P = 0.0005) because both canopy and density covary with altitude, a more important predictor variable (Pearson Correlation r = –0.41 between altitude and canopy cover, –0.5 between altitude and seedling density, –0.43 between altitude and sapling density; P <0.0005 for all). In such a situation, variance explained by a less important variable in bivariate regression is captured by a more important variable when the variance in the dependent variable is explained simultaneously by multiple explanatory variables.

However, given the established relationship between canopy cover and density of seedlings and saplings (Neyisci

1993, Spanos et al. 2001, Bonnet et al. 2005) and our observation in the field that seedlings and saplings grew more densely in canopy gaps, the more likely explanations are the following. The non-parametric test (tree regression analysis) that we used may not have been powerful enough to detect the relationship between canopy cover and density of young plants. Another possibility is that the rule we adopted to determine the best model (tree) was very conservative yielding a sub-optimal tree that could not capture all the important predictor variables. Whereas Breiman et al. (1984) proposed both a minimum cross-validation rule and a 1 standard error (SE) rule, there is a lack of clear reason on the choice of the rule for determining best model. The minimum cross-validation rule assumes that the optimal tree is the one with the smallest cross-validation error; the 1-SE rule states that the optimal tree is the smallest tree within 1 standard error of the minimum cross-validation error. The negative relationship between cross-validation error and tree complexity (cross-validation error declines as tree grows until a minimum error is reached) makes it likely that 1 SE rule can yield a simpler model than minimum rule does, especially when multiple predictor variables exist. In such a situation, the 1 SE rule could yield sub-optimal trees. However, to avoid any potential false positives and to remain consistent with many studies in the practice of model selection, we applied the more conservative 1-SE rule, which might have been unable to capture some of the important predictor variables, such as canopy and moisture.

The quantity and duration of soil moisture depend on aspect because of the stark difference in the amount of sunlight received by north and south aspects (Parker 1991). As southern aspects receive more intense insolation than northern aspects, the northern aspects retain more soil moisture, which provides more suitable conditions for seed germination, seedling establishment and regeneration of *P. wallichiana*. The northern slopes in our study sites are moister – and have more canopy cover – than the southern slope; these two factors both have a positive influence on seedling germination. Some plots in moist gullies on the southern aspect also had higher seedling abundance than other dryer plots on the same aspect which were relatively dry. In contrast to the density pattern that we observed, Schickhoff (1996) found that *P. wallichiana* is more abundant on the relatively dry south-facing slopes than on the north-facing slopes in the Kaghan Valley of northern Pakistan.

Fire damage to forests has been reported to accelerate regeneration of *Pinus* species and increase seedling density; this is the case, for instance, with *P. brutia* on the Thasos Island of north Greece (Spanos et al. 2001) and *P. ponderosa* in the Black Hills of South Dakota, USA (Bonnet et al. 2005). Schweinfurth (1957) and Stainton (1972) have argued for the significant role of fire in the maintenance of *Pinus* forests in the Himalaya. Fire damage that occurred about 35 years ago on the north-facing slopes of our study site probably changed the course of forest regeneration. However, without detailed historical data, we are unable to relate that event to today's state of forest regeneration.

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Simulating farm income under the current soil management regime in the mid-hills of Nepal

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Farmers in the mid-hills of Nepal follow diverse farming systems. The peri-urban area of this region, where population density is higher, faces several problems in farming. While hills suffer from erosion because they are erodible, the peri-urban areas face the problem of decline in factor productivity, particularly in intensively cultivated farmlands. The present study is concerned with simulating farm income on a regional scale based on soil management practices. Spatial explicit simulation shows that the loss of farm income due to degradation is substantially higher in hills while it is lower in valley bottoms. Strategy formulation and testing in the spatial environment indicates that Geographic Information System is an appropriate methodological tool for simulating the consequences of particular interventions.

Key words: Mid hills, Nepal, spatial modeling, soil quality index, farm income

The mid-hills cover about 43% of Nepal's land area (Shrestha 1992) and accommodate 46% of Nepal's population. There is a great diversity of land use due to variations in topography, population density and market demand (Bhatta 2010a). The fulfillment of subsistence requirements has for centuries been the primary objective of the majority of the farmers in the mid-hills (Carson 1992; Brown 1997). However, in recent decades market-oriented production has emerged as a key driving force for land-use intensification in the densely populated urban fringes of Nepal (Brown and Shrestha 2000). While subsistence farming is characterized by the integration of livestock and forestry with agriculture and traditional modes of production, intensification is characterized by double or triple crop rotations, expanded cultivation of vegetable cash crops, and the imprudent use of agro-chemicals (Bhatta 2010a).

Road access, along with proximity to input markets, is the main catalyst for expansion of commercial farming (Brown 2003; Brown and Shrestha 2000) and consequent use of agro-chemicals (Bhatta and Doppler 2011). In the early 1980s agro-chemicals first appeared in newly accessible areas and their use quickly accelerated (Pokhrel and Pant 2008). The environmental and health costs of inorganic farming have by now been widely felt in Nepal, raising awareness of the issue of sustainability (Bhatta et al. 2009); meanwhile, agriculture based on organic practices and balanced application of inputs on family-owned farms in the peri-urban and rural areas has shown a great deal of resilience (Sharma 2006). This is because sustainable farming addresses many environmental and social concerns and offers innovative and economically viable opportunities for growers, laborers, consumers, and other stakeholders, as well as policymakers.

The problem of soil degradation exists in almost all parts of the mid-hills of Nepal, but the severity varies depending on different factors. Cultivation of the sloping marginal hills leads to severe soil erosion, while the scars of the green revolution are visible in the urban and peri-urban flat lands. Bio-physical factors such as variations in weather, landforms, soil types and resource availability (Verbung et al. 2004) as well as socio-economic factors such as social structure, family composition and needs, have combined with economic opportunities, technological availability and political systems to affect land use evolution (Briassoulis 2000).

Spatial methodologies are commonly used for the analysis of socio-economic phenomena and their distribution along the spatial gradient (Bhatta et al. 2009; Codjoe 2007; KC 2005; Evans and Moran 2002; Schreier and Brown 2001; Bowers and Hirschfield 1999; Joshi et al. 1999). The present study integrates micro-surveys in a Geographic Information System (GIS) in order to model the current situation and predict future economic viability of family farms, assuming the persistence of prevailing soil management practices.

Materials and methods

Location and physical aspects of the study area The study was undertaken in the Lalitpur and Bhaktapur Districts, which have biophysical and socio-economic characteristics typical of the mid-hill region of Nepal (**Figure 1a**). The low-lying flat plains of this region are characterized predominantly by the

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rice-wheat cropping pattern, while cultivation in the rainfed uplands is typically based on maize. Both of these cropping patterns are exhaustive in nature. Since most farmers in the mid-hills lack irrigated land, they must rely on maize as their major food source. Currently, farmers are producing several species of vegetables, both for *in situ* consumption and for market. However, commercial vegetable farming based on widespread use of agro-chemicals has had negative repercussions on agro-ecology.

The peri-urban part of the study area is located mostly in the Bhaktapur District and partly in the Lalitpur District, while the rural area is located in the hilly part of Lalitpur District. **Figure 1b** shows that the study area is characterized by an altitudinal gradient ranging from 900 to 2500 meters above sea level (masl). Elevation ranges from 1500 to 1800 masl cover much of the area, with only negligible land surface at less than 1000 masl.

Slope of the study area, derived from digital elevation model (DEM), is expressed in percent. Slope at a given grid cell is estimated from elevation of the surrounding eight grid cells. The following grid consists of nine grid cells labeled “A” through “I”. If “*a*” represents elevation of the gird cell “A”, “*b*” elevation of the grid cell “B”, and so on, then the slope for cell “E” (central cell) in the following grid is determined as follows (equation [i]):

A	B	C
D	E	F
G	H	I

$$\% \text{ Slope} = \sqrt{(\Delta z_x^{2}+\Delta z_y^{2})} \quad \text{...} \quad \text{(i)}$$

where, $\Delta z_x^{2} = [(a+2d+g)-(c+2f+i)] / (8 \times \text{cell size})$

$\Delta z_y^{2} = [(a+2b+c)-(g+2h+i)] / (8 \times \text{cell size})$

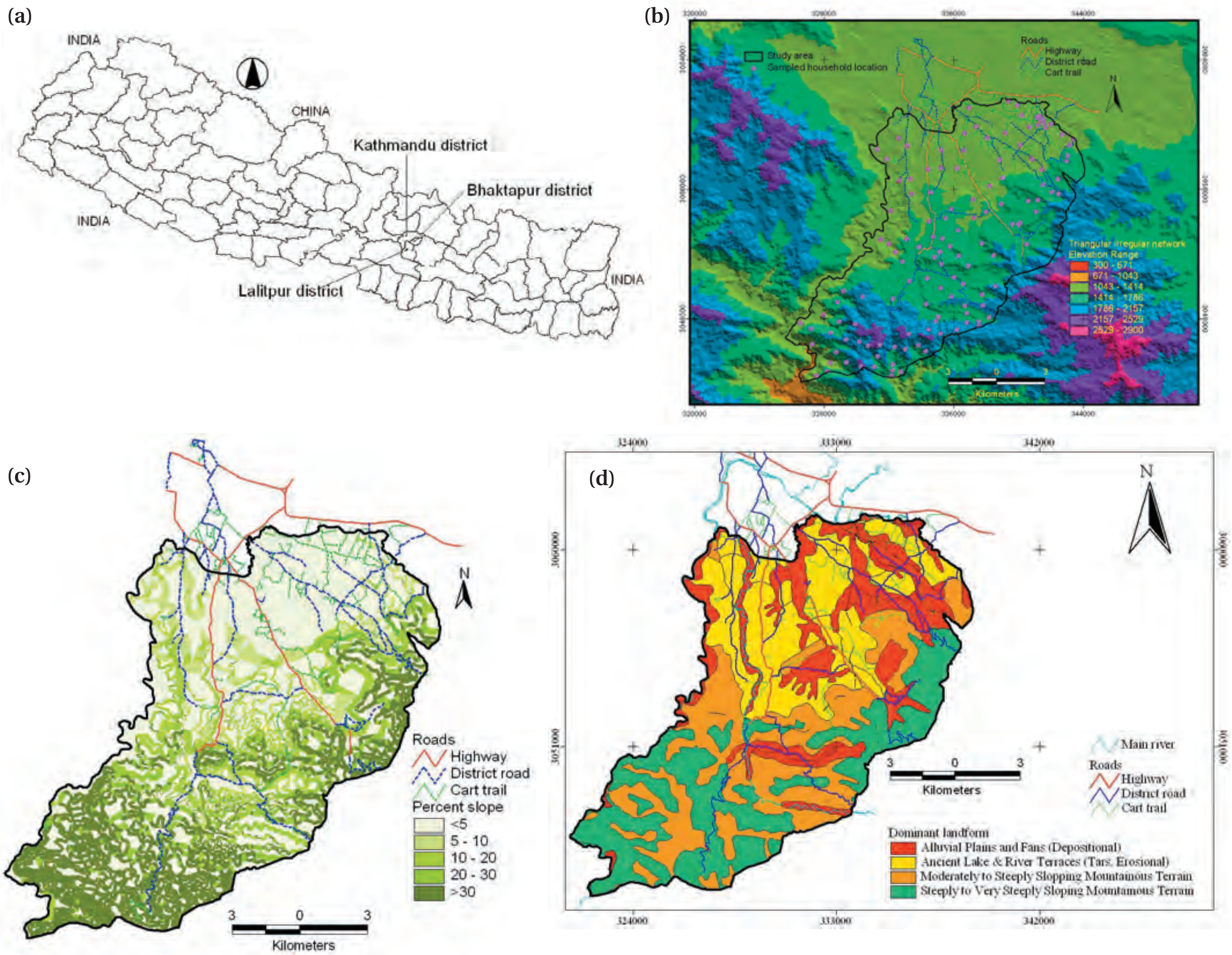

Figure 1. The study area: (a) map of Nepal showing the Lalitpur and Bhaktapur Districts, (b) digital elevation model of the study area, (c) slope (%) in the study area, and d) dominant landforms

A sizeable part of the study area is flat or nearly flat (0 to 5%); most of the land has a steep slope (>30%) (**Figure 1c, Table 1**). The rural hills, in general, have steeper slopes than do the urban and peri-urban areas. Slope along with fragile landscape leads to severe soil erosion in hill farming systems throughout the country (Brown and Shrestha 2000).

Four dominant landforms are found in the study area (**Figure 1d**). Alluvial plains and fans, generally with a slope of less than 5%, are composed of deposits from floodwater or runoff and tend to be rich in nutrients. Most of the Kathmandu Valley bottom is comprised of these formations. Another group of landforms, the ancient lake and river terraces (locally referred to as *tars*), are formed by water erosion; they have a gentle slope. The forth type of landform is composed of moderate and steep slopes which are prevalent primarily in hilly part of the study area.

Sampling and the data The study was based on a cross-sectional study of 130 farms. Ninety households were selected through spatial sampling; the remaining 40 were selected at random. Spatial sampling was adopted in rural and peri-urban areas because little information was available about these scattered households.

Household data were collected using a standard questionnaire prepared subsequent to a pilot study and administered through personal interview. The spatial data were collected from already available maps. These data include elevational contours, dominant landforms, soil types, roads and other infrastructure.

Integrating socio-economic data into the GIS This paper represents an attempt to develop a model by which farm income over years can be simulated based on a degradation scenario. The methodological framework entails integrating micro-survey data into a spatial environment. Farm income was calculated taking into account many facets of the farm economy, including production costs and market prices of crops. Farm income was integrated into the GIS database using Global Positioning System (GPS). Prior to spatial integration, the significance of the farm income variable was subjected to a test of spatial autocorrelation using Geary's Ratio and Moran's I. It was then subjected to spatial interpolation using Inverse Distance Weighting (IDW) to generate the output grid surfaces in which the value of each cell was 25 meters by 25 meters. The interpolation was performed based on the values of 12 neighboring sample points and their distance to the point of estimation. A linear trend in the sample data was assumed in the IDW.

We produced the digital elevation model (DEM) incorporating terrain parameters such as slope and elevation. Cost-distance analysis and dominant landform with land management practices along the spatial gradient were incorporated in the regional spatial model. Cost distances from farms to market center were calculated using the GIS-based cost weighted distance model (ESRI 1997). Biophysical variables such as road infrastructure and slope were considered in the cost-distance modeling. This technique is based on the idea that a relative "cost" can be associated with moving across each cell in a map (ESRI 1992).

Table 1. Area distribution under different slopes in the study area

Slope range (percentage)	Area (ha)	Percentage of total
<5	8708	48.6
5–10	1338	7.5
10–20	1450	8.1
20–30	1945	10.9
>30	4463	24.9
Total	17903	100.0

The cost of moving across a cell is calculated as the cell size (in meters) times a weighting factor based on the quality of the road and associated factors of the cell such as slope. The least-cost model evaluates the cost of moving between two designated source areas (from household location to market center) by calculating, for each cell, the cumulative weighted distance between the cells and the two sources.

Soil quality weighting In preparing a comprehensive soil quality weighting for the study area, we considered dominant landforms available and four soil management practices commonly followed by the farmers.

Landforms are composed of typical varieties of soil with varying production potentials. Dark soils containing alluvial deposits, for instance, have good capacity to retain water and to supply nutrients (Singh et al. 2007), the essential requirements of the majority of crops (Rajbhandari and Bhatta 2008). Lands rich in this first type of soil were given a high score. The second group is composed of soils around ancient lakes and river terraces, which have a higher rate of erosion than the first class. These lands are composed of hills with narrow valleys and elongated ridges; predominantly occupied by soil that is well to excessively drained, loamy skeletal in texture, slightly acidic (pH 5.2 to 6.9), with a shallow rooting depth (Singh et al. 2007). Lands dominated by these types of soils grow food crops successfully, but yields are not comparable to those of the higher-weighted class described above.

A third landform type is composed of mountainous terrains with moderate slope, generally suitable for subsistence farming; the cost of land management is greater here than on flat lands. This landform was given lower weight than the classes discussed above. The fourth landform group is composed of mountain terraces with steep to very steep slopes, thin soil with stony subsoil; they are subject to severe erosion by both wind and water (Müller-Böker 1991). The cost of land management is excessively high due to the rugged terrain. This group of lands has been given the lowest score. The difference between scores for alluvial flat lands and for mountainous terrain was calculated using the gross margin of rice. The ratio of the gross margin of rice in both classes is approximately to 1.5. The difference in the productive potential of two landforms composed of alluvial soils is very slight. They were, therefore, given higher values with narrow difference. Similarly, in weighting mountainous terrains with moderately steep and very steep slopes, we considered the gross margin of maize; the ratio between the gross margins of maize in steep and very steep slope lands turned out to be 1.2. Therefore, we assigned a value of 1.70 to steep land and 1.40 to very steep land (**Table 2**).

Soil management practices Fertility management practices followed currently are balanced fertilizer application, use of agro-chemicals only, use of manure only and unbalanced application of manure and fertilizers.

Balanced fertilizer application

This refers to soil management practices employed by organic growers around the peri-urban areas. Farmers using these practices apply organic manure and other locally available resources. They also follow other fertility management practices such as intercropping, terracing and application of farm waste to crops. Pest control is generally implemented by means of local materials and botanicals.

Use of agro-chemicals only

Commercial vegetable growers in the peri-urban areas follow this practice. Most farmers using agro-chemicals are near input markets. Generally, exhaustive crops and their rotations are followed. Farmers experience decline in partial factor productivity of fertilizers and pesticides in their farmlands.

Unbalanced application of manure and agro-chemicals

This is a kind of intermediate practice and is followed by some farmers in the peri-urban area. Farmers apply both organic manure and inorganic fertilizers. Although farmers do understand the value of organic manure in agriculture, chemical fertilizers are applied in concentrations so high that the buffering capability of the manure is overwhelmed.

Use of farm manure only

Farmers in rural areas follow this practice, in which crop nutrients are derived solely from locally-produced manure. Some farmers apply inorganic inputs, but the amount applied is so negligible that we would not characterize the practices as "inorganic farming." Rather, this mode of agriculture is more often referred to as organic by default or organic by neglect. The quantity of nutrient supplied to the crops is far below the crops' requirements, and the organic manure applied in the field is not enough to prevent soil erosion. Therefore, this form of soil management is not considered sustainable.

Balanced input application is important for good yields, and is considered one of the key components of sustainable agriculture. Consequently it is assigned a high value (2.00), followed by intensive land management based on inorganic inputs (1.90). Application of higher amount of inorganic fertilizer can make good yields likely, but a small amount of farm manure applied is unable to improve edaphic environment. Therefore, unbalanced application of organic manure and inorganic fertilizers this soil management practice is weighted at 1.80. The last category of management is traditional subsistence farming. Manure application is not enough to provide the nutrients required for a good yield. Lands managed in this way are accorded a low weighting (1.50).

After assigning a weight to each farm based on dominant landform and land management, we produced a map representing these characteristics using GIS overlay

Table 2. Land quality weighting based on landforms and farmers' practices of soil fertility management under current and the future scenarios (degradation scenario)

Landform	Land management	*Current scenario* Landform	Management	Combined	*Soil degradation scenario* Degradation	Combined
Alluvial plains and fans (depositional)	Unbalanced	1.40	1.80	2.52	1.62(10)	2.27
	Manure	1.40	1.50	2.10	1.35(10)	1.89
	Balanced	2.00	2.00	4.00	2.00	4.00
	Chemical	2.00	1.90	3.80	1.77(7)	3.54
Lake and river terraces (tars, erosional)	Unbalanced	2.00	1.80	3.60	1.71(5)	3.42
	Manure	2.00	1.50	3.00	1.50	3.00
	Balanced	1.90	2.00	3.80	2.00	3.80
	Chemical	1.90	1.90	3.61	1.71(10)	3.25
Mountain terrains with moderate slope	Unbalanced	1.90	1.80	3.42	1.67(7)	3.17
	Manure	1.90	1.50	2.85	1.46(3)	2.77
	Balanced	1.70	2.00	3.40	2.00	3.40
	Chemical	1.70	1.90	3.23	1.58(15)	2.69
Mountain terrains with steep to very steep slope	Unbalanced	1.70	1.80	3.06	1.62(10)	2.75
	Manure	1.70	1.50	2.55	1.35(10)	2.30
	Balanced	1.40	2.00	2.80	2.00	2.80
	Chemical	1.40	1.90	2.66	1.58(15)	2.21

Note: Values in the parentheses indicate the reduction in the score due to degradation by a given percentage technique. The generalized formula to calculate the combined index is:

$$(SQ_{present})_i = (W_l \times W_{mp})_i \ldots\ldots \text{(ii)}$$

$$(SQ_{future})_i = \{W_l \times (W_{mp} - W_{mp} \times \%R)\}_i \ldots\ldots \text{(iii)}$$

where each of the following values is associated with the i^{th} cell: SQ_i, soil quality; W_l, weight attributed to the landform; W_{mp}, weight associated with the current soil management; $\%R$, reduction in weight due to soil management practices.

Following equation (ii), altogether 16 classes were formed in which the highest weight (4.00) was attributed to alluvial plain lands with balanced fertility management, while the lowest weight was assigned to steep-sloped mountainous terrains in the only fertilizer applied is locally-produced manure (2.10) (**Table 2**).

Results and discussion

Soil degradation scenario and land quality weightings Practices such as continuous deployment of an exhaustive cropping pattern without prudent use of chemical fertilizers, abstinence from conservation measures and multiple cropping, and exploitation of marginal lands can exacerbate the problem of fertility degradation (Brown and Shrestha 2000). The single greatest cause of declining crop production is unbalanced fertilization (Rattan and Singh 1997). Unbalanced fertilizer application has led to a chronological emergence of macronutrients such as phosphorus and potash (P and K) and micronutrients such as zinc, sulfur and manganese (Zn, S and Mn) deficiencies. Even balanced application of macronutrients devoid of organic materials has been implicated in the deterioration of the physical, chemical and biological health of soil (Rattan and Singh 1997). Most farmers have realized that prolonged overapplication of fertilizers is not sustainable in the medium to long run (Joshi et al. 1996).

A decline in the partial factor productivity of nitrogen is generally due to a decrease in the nitrogen-supplying capacity of intensively cultivated lowlands (Cassman et al. 1994). A series of long-term experiments initiated in India and Nepal indicated the superiority of organic materials such as *Sesbania aculeata* (Mandal et al. 1992; Singh et al. 2000; Kundu and Samui 2000), FYM (Prasad and Sinha 2000) and residue (Singh et al. 2000; Prasad and Sinha 2000; Gami and Sah 1999; Bhatta and Subedi 2006) in enhancing soil quality and maximizing crop yields.

The rate of degradation in fertility also varies according to landform. For instance, the rate of soil decline is lower on plains than in hilly areas because of the compounding effects of steep slope and land structure. The decreasing use of organic matter and the land use shift from traditional subsistence farming towards intensive vegetable farming in the hill terraces will exacerbate land quality degradation in the future (Tiwari et al. 2009). Nevertheless, factor productivity on plains is declining because of the excessive use of agro-chemicals and continued monocropping of exhaustive crops (Bhatta 2010a). Soil acidification caused by urea is a common concern in most intensively cultivated areas (Brown and Shrestha 2000). Therefore, farmland with balanced fertilizer management (application of substantial quantities of organic manure along with a small proportion of inorganic fertilizer, as well as legume intercropping, for example) would have almost same quality weighting in the future. By contrast, production practices that are heavily dependent on agro-chemicals will result in fertility decline (Bhatta 2010b). While in the alluvial lands the reduction due to intensive agro-chemical use is expected to be 5%, on river terraces with erosional land where agro-chemicals are abused, the fertility decline is assumed to be around 10%.

Table 3. Model summary of the multiple regression (dependent variable: farm income in †NRs·ha^{-1})

Parameters	B	β	SE(B)	t value
Constant	–110504		2273	–57**
Cost-distance (minute)	–2615	–0.25	19	–135**
Land quality	163200	0.56	0.05	301**
R^2 = 61%, F-statistics (2, 282214) = 212500 (p<0.01)				

Note: ** significant at 1% level; †73 NRs = 1$

Results provided by running a soil erosion assessment model (Morgan et al. 1984) in the GIS environment show that annual soil loss rates are highest (up to 56 tonnes·ha^{-1}·year^{-1}) on terraced slopes in hilly areas. Erosion from cultivated and grazing lands is a serious problem, and marginal upland agricultural sites are prone to a higher erosion rate (Brown and Shrestha 2000). If farm production is based solely on agro-chemicals, we would assign a weighting 15% lower than would otherwise be attributed to that land. Similarly, with unbalanced land management practices, weighting reductions of 5%, 7% and 10% from the basic land quality are attributed to farmland on alluvial plains, river terraces and moderate to sloping terrain, respectively. In the alluvial plains, farming based solely on the application of an ample amount of farm manure is an ideal strategy to restore fertility and produce an acceptable yield. No reduction in weight is considered under this management practice; it is weighted at the same value. The amount of manure applied by the farmers, however, cannot meet the nutrient requirements of crop plants and cannot prevent soil erosion to same extent it would on river terraces and higher sloped lands. Therefore, such practices entail weight reductions of 3% and 10% from the baseline for river terraces and sloped lands, respectively.

For the purposes of our calculations, the prices of inputs as well as outputs were held constant: we assume that the impact of future inflation will be roughly equal on both sides of the ledger. It is also assumed that there will be no technological change in crop production for the time span considered and that farmers will use the same amount of inputs as in 2007, our base year. Consequently, land management is the single largest factor influencing the performance of production systems in our projections.

Following equation (iii), alluvial plains with balanced fertility management are accorded the highest quality score followed by river terraces with balanced application and alluvial plains where agro-chemicals are used. Under degradation scenario, there is no effect on soil quality under balanced fertilizer application while there has been substantial decrement in soil quality on the sloping landforms (**Table 2**).

Base model

The GIS-based multiple regression model, in which farm income is the dependent variable and land quality and cost-distance to primary market are the independent variables, shows significant trends. All variables in the model have the expected direction of relationship (**Table 3**). The predictive power of the model is 61%. Higher predictive power of the model signifies its better fit in simulating farm income. A unit increase in cost-distance reduces farm income by NRs 2,615; a unit increase in land quality, *ceteris paribus,* increases farm income by NRs 163,200.

Interpolated observed farm income (NRs·ha^{-1}) along the spatial gradient is shown in **Figure 2a** and estimated farm income using an emperical regression model are shown in **Figure 2b**. Both observed and estimated income have similar trends in accessible areas, while there is a mixed tendency at higher altitudinal gradients. This is because the regresion function underestimates income in the inaccessible areas. Similarly, both of the figures show declining farm income as one goes towards the rural setting from peri-urban areas.

Simulated model under land degradation scenario

Farm income under the soil degradation scenario was estimated using multiple regression, and the resulting functional form is presented in equation (iv). We used estimated farm income under the current regime (as shown in **Figure 3a**), and farm income under the degradation scenario was deducted from current income while the difference was taken as the impact of degradation. The explanatory power of the independent variables is slightly higher (R^2=65%) under future scenario (degradation situation) than that under the current situation (R^2=61%). All of these coefficients are highly significant in predicting the changes in farm income per hectare (ha). One unit increase in the cost distance, in terms of travel time in minutes, would reduce farm income by NRs 2,244 while a unit increase in land quality weighting would increase farm income by NRs 174,400, as given by the following equation:

$$Y = -135692\ (-73^{**}) - 2244\ X_1\ (-129^{**}) + 174400\ X_2\ (376^{**}) \quad \text{(iv)}$$

*(values in parentheses indicate t-statistic; ** indicates statistically highly significant with p<0.01)*

R^2= 0.65, F stat (2, 282212) = 260500 (p<0.01)

where Y is farm income, X_1 cost distance and X_2 land quality.

Figure 3a shows estimated farm income (NRs·ha^{-1}) regressed by assumed land quality weighting and cost-distance to primary market. **Figure 3b** depicts loss in farm income due to the future scenario (degradation situation) as compared to the present situation. The current situation shows three distinct areas with respect to farm income, viz.: high, medium and low income zones, the high-income zone being located in the peri-urban areas while medium- and low-income zones are located in rural areas.

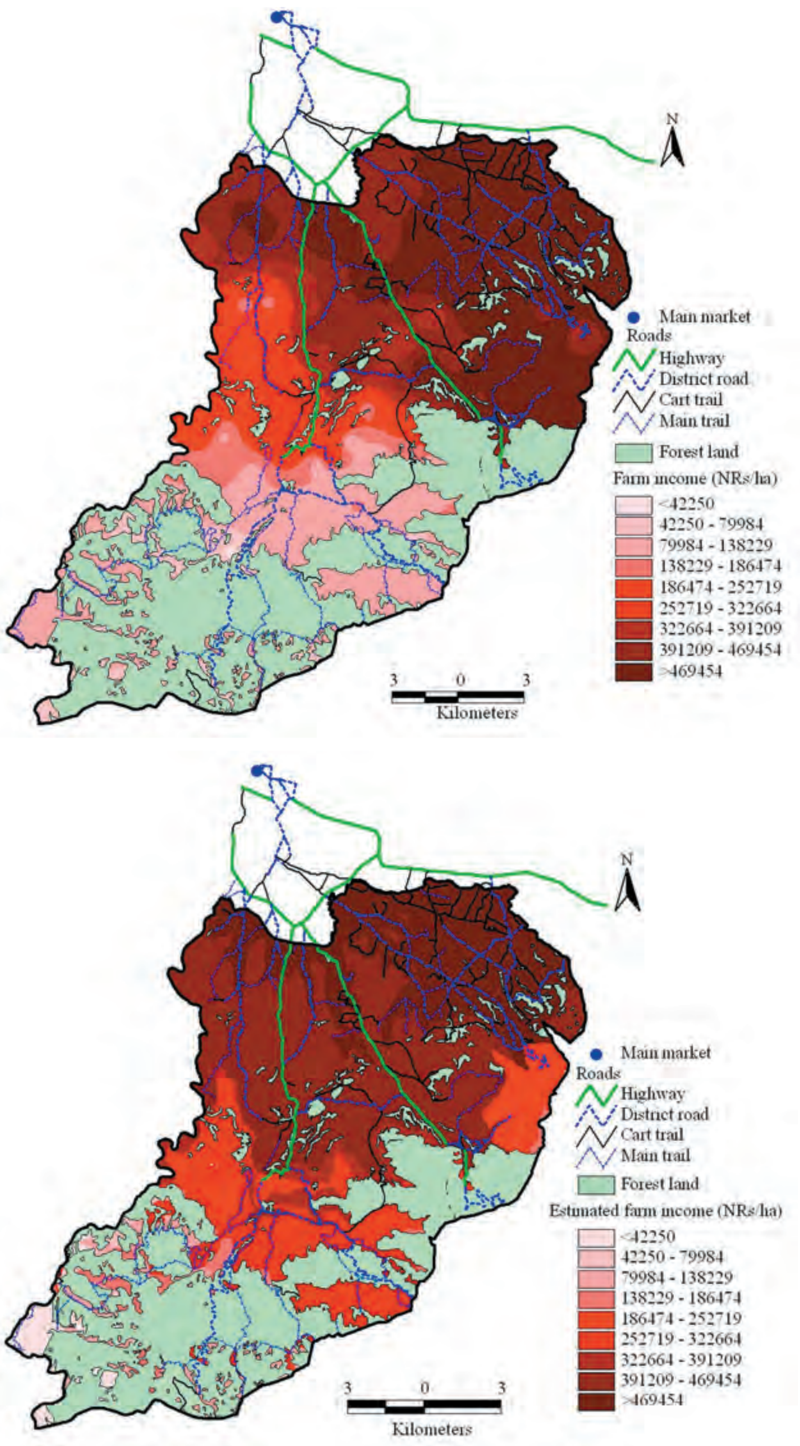

Figure 2. Farm income (NRs·ha^{-1}) based on current scenario: (a, top) as obtained from the household survey, and (b, bottom) as estimated using regression model

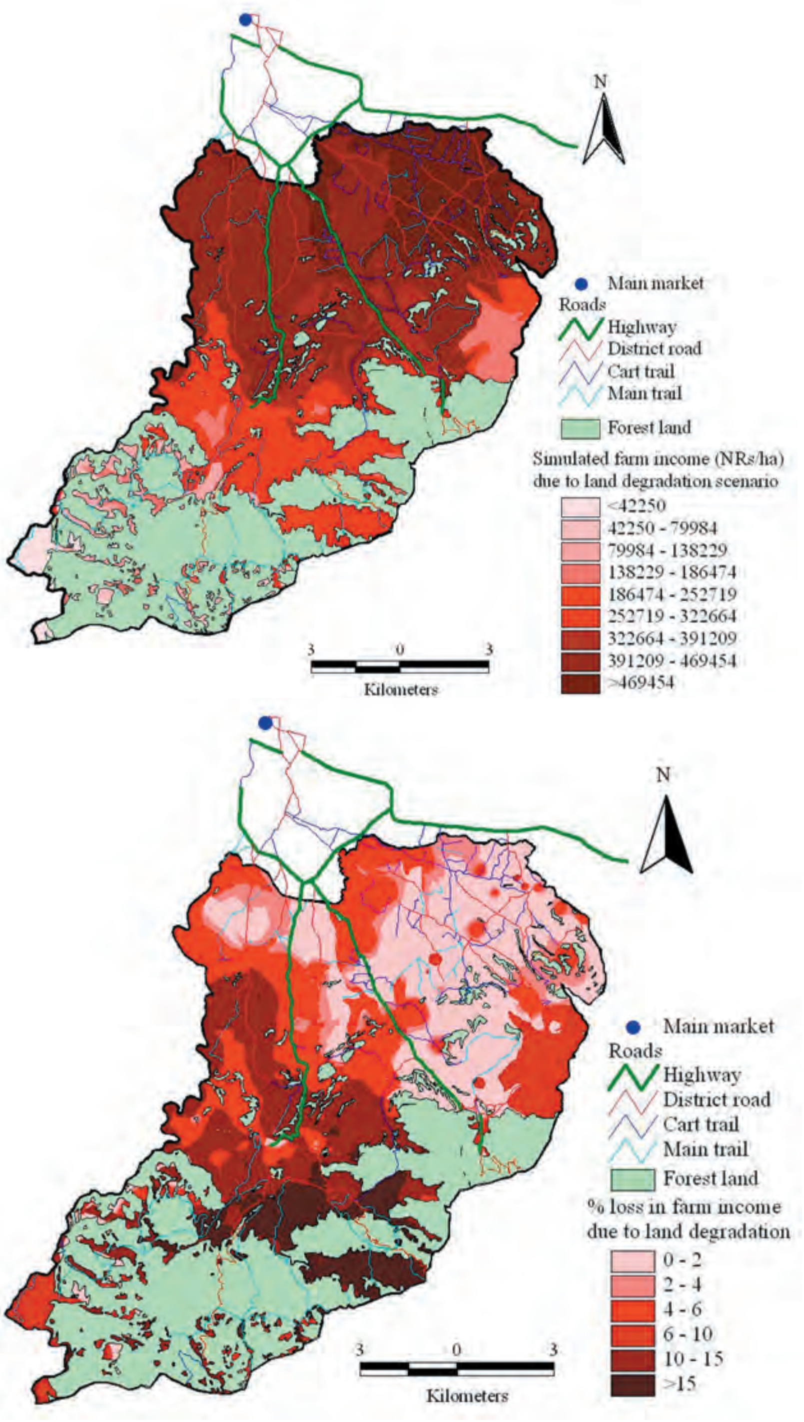

Figure 3. Farm income (NRs·ha^{-1}) associated with the future strategy of land degradation simulated using a spatial explicit model: (a, top) simulated farm income (NRs·ha^{-1}) under soil degradation scenario, and (b, bottom) impact of soil degradation on farm income (% loss)

The loss of farm income due to degradation is substantially higher in the low income zone (mainly in the hills), where it goes higher than 15%, while it is very small (0–2%) in high-income areas. Within the high-income accessible region, there is almost negligible loss of income, particularly where farmers follow organic practices (apply ample amount of organic manure), whereas income loss goes as high as 10% in the commercial inorganic farming area. This is due to the fact that farming in this zone is based solely on inorganic inputs whose continued use would reduce soil quality in the future. The higher loss of income in the rural area is basically attributable to low quality of the land associated with high erosion exacerbated by steep slopes.

The rural area is characterized by subsistence farming with poor standard of living. Income in most of remote areas ranges from less than 42,250–1,864,747 NRs·ha^{-1}·year^{-1} and a loss of 10–15% income would have a substantial impact on the standard of living. This shows that rural life depends heavily on local resources, especially soil, and their degradation would have enormous effects on the income generation potential of farmers.

Conclusion

Four dominant practices of soil fertility management are assumed in this study. The baseline spatial explicit model shows a clear variation in farm income along the spatial gradient. Balanced application is considered a sustainable way of enriching soil and hence restoring its fertility over time. Relatively inaccessible rural areas have lower farm income than peri-urban areas. Farm-families living in the higher altitude relatively inaccessible areas have a lower standard of living and they are highly dependent on farming for their subsistence needs. The low lying valley hinterlands with good road access and other infrastructure are more tractable in terms of agricultural enterprise, but agro-ecological degradation should be taken seriously. GIS-based socio-economic analysis and modeling is a key approach to the study of complex phenomena and formulation of policies for future development.

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Research paper

Optimization of RAPD-PCR conditions for the study of genetic diversity in Nepal's *Swertia chirayita* (Roxb. Ex Fleming) H. Karst

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Of the 30 species (including five varieties) of the genus *Swertia* in Nepal, nine have been reported to possess medicinal properties. Among these, *S. chirayita* is the most valuable species, with high demand in domestic and international markets. Nepal's *S. chirayita* and related species are being recklessly exploited for commercial purposes. Two problems that have emerged with this lucrative market are (a) adulteration and fraudulent labeling of *S. chirayita*, and (b) depletion of *S. chirayita* and allied species from their natural habitats. To address the problem of adulteration and conservation, we studied molecular genetic diversity in *S. chirayita* populations and developed a molecular diagnostic tool for the purposes of authentication. We studied intra-specific genetic diversity in *S. chirayita* using Polymerase Chain Reaction (PCR)-based Random Amplified Polymorphic DNA (RAPD) technique. As a preliminary step, we identified optimal RAPD-PCR reaction and cycling conditions by varying PCR reaction parameters such as concentration of template DNA, $MgCl_2$, dNTPs, primer, Taq DNA polymerase and RAPD-PCR programs. The optimized PCR reaction and cycling conditions were then used in subsequent RAPD profiling experiments for the study of genetic diversity within *S. chirayita* populations from various geographical locations. Genetic diversity characterization of *S. chirayita* populations at the molecular level would furnish information with significant applications in the conservation and sustainable utilization of *S. chirayita* and its allied species in Nepal.

Key words: Polymerase Chain Reaction, Random Amplified Polymorphic DNA, DNA fingerprinting, genetic diversity

Globally, genus *Swertia* is represented by approximately 100 species (Willis 1996). Of the 30 species, including five varieties, that have been reported in Nepal (Press et al. 2000), nine are being traded for their medicinal properties, viz.: (1) *S. chirayita* (Roxb. Ex Fleming) H. Karst, (2) *S. angustifolia* Buch. – Ham. Ex D. Don, (3) *S. tetragona* Edgew, (4) *S. racemosa* (Griseb) C. B. Clark, (5) *S. cilaita* (D. Don ex G. Don) B. L. Burtt, (6) *S. dilatata* C. B. Clarke, (7) *S. multicaulis* D. Don, (8) *S. alata* (Royle ex D. Don) C. B. Clarke, and (9) *S. nervosa* (G. Don) C. B. Clarke (Joshi and Joshi 2001, Rajbhandari 2001). Among these, *S. chirayita* is considered superior to all the others in quality and is in high demand for trade (Pant 2011). *S. chirayita* is distributed in the hills of eastern, central and western Nepal at an altitude of 1200–3000 m in open forests and shady habitats. Bhattarai (1996) reports the species in 40 districts of Nepal; Barakoti et al. (1999) reports distribution in 54 districts.

Eighty to 90% of the total harvest is exported to India as a crude drug and about 9% to China, Malaysia, Singapore, Germany, Italy, France, Switzerland, Srilanka, Bangladesh, Pakistan and the United States. Nepal consumes only 1% of the total harvest and supplies 45–50% of the world's total volume (Joshi and Dhawan 2005). It has been reported that more than 27.2 tons of *S. chirayita* (valued at more than 2.3 million Indian rupees) was exported from Nepal during 1997–1998 (Joshi and Dhawan 2005).

The medicinal properties of *S. chirayita* are attributed to a number of chemical compounds with therapeutic value; these include chiratanin, gentianine, amarogentin, amaroswerin, and several xanthone, iridoid, triterpenoid and glycoside derivatives (Bajpai et al. 1991, Chakravarty et al. 1991, Benerjee et al. 2000, Joshi and Dhawan 2005). The whole plant is used in crude form, and it is also used to manufacture various ayurvedic, herbal, and allopathic medicines. The importance of *S. chirayita* as a multipurpose drug was perceived already in ancient times (Kiratatikta 2001). In "Ayurvada," *S. chirayita* is said to contain anti-cancerous properties (Chiraito *Swertia chirayita*, www.ansab.org). In Ayurvedic preparations, it is used as antipyretic, hypoglycemic, antifungal and antibacterial agent (Joshi and Dhawan 2005). Although the entire plant has medicinal

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properties, in traditional medicines the root is considered the most powerful part and is used in the treatment of chronic and malarial fever, joint pain, ulcers, cough, cold, asthma, scabies, leucoderma etc. (Chiraito *Swertia chirayita,* www.ansab.org). One of the constituents, Swerchirin, has been found effective in lowering human blood sugar level (Bajpai et al. 1991, Saxena et al. 1993) through the stimulation of insulin release from the islets of Langerhans. Its anti-leishmanial property has been studied in hamster model (Medda et al. 1999), and its anti-inflammatory and hepato-protective effects have been studied in mice model (Islam et al. 1995, Mukherjee et al. 1997).

S. chirayita has been listed as one of the top priority medicinal plants of Nepal as well as of Asia (DPR 2005). The great demand, both in Nepal and abroad, has led to its rapid depletion in the wild. Because of the availability of multiple species of *Swertia* in Nepal, a number of other allied species are also being traded. This trend of adulteration, from human health safety viewpoint is a malpractice. The World Conservation Union (IUCN) has categorized this species as 'Vulnerable' (IUCN 2004). An urgent need has been felt to conserve its existing diversity in different geographical gradients of Nepal while sustainably utilizing it for the economic benefit.

Patterns of genetic diversity in plants can be studied by analyzing morphological, anatomical, embryological, biochemical and molecular traits (Weising et al. 1995). Historically, the method of choice has been to focus on morphological characters as the basis on which different plant species are identified and classified into different taxonomical hierarchical groups. However, the classical tools are being increasingly complemented by advanced biochemical tools (focusing on isozymes, allozymes, seed proteins, and secondary metabolites) as well as molecular tools based on restriction fragment length polymorphism (RFLP) and hybridization, polymerase chain reaction (PCR), and DNA sequencing (Murphy et al. 1996, Judd et al. 2002, Chawla 2003). Various PCR-based molecular marker techniques, have already been widely used for genetic diversity analyses, identification of genotypes in genebank management and molecular phylogenetic studies and development of species diagnostic protocols; these techniques include those based on Random Amplified Polymorphic DNA (RAPD), microsatellites or Simple Sequence Repeats (SSRs), Inter-Simple Sequence Repeats (ISSRs), Amplified Fragment Length Polymorphism (AFLPs), and Internal Transcribed Spacers (ITS) sequences (Edwards 1998, Matthes et al. 1998, Wetzel et al. 1999, Yasuda et al. 2002, Shrestha et al. 2003, Joshi et al. 2004, Shrestha et al. 2005, Qian et al. 2006, Joshi and Dhawan 2007). RAPD analysis allows detection of polymorphism in closely related organisms (e.g., different populations of single species or individuals within a population) and therefore provides a powerful tool for tasks such as population and pedigree analysis, genetic diversity and molecular diagnostic development (Micheli et al. 1997).

In the present study, an attempt has been made to optimize RAPD-PCR reaction and cycling parameters for the study of genetic diversity in *S. chirayita* collections from four districts of Eastern Nepal (Ilam, Terhathum, Dhankuta and Sankhusabha), two districts of Central Nepal (Lalitpur and Kathmandu) and one district from Western Nepal (Kaski). PCR-based molecular genetic diversity studies furnish valuable information regarding genetic diversity in *S. chirayita* populations from various geographical locations and their inherent genetic relationship. A study of molecular genetic diversity in various populations of *S. chirayita* would not only help us understand the evolutionary aspect of the species but also provide valuable insights for its conservation and sustainable utilization. Such a study would also identify a number of taxonomic units for conservation purposes and would enable the linkage of genetic diversity information with data regarding chemical properties (Alam et al. 2008). In addition, since molecular tools hold great promise for diagnostic development (Shrestha et al. 2005, Qian et al. 2006, Shrestha et al. 2010) and medicinal plant authentication (Joshi et al. 2004, Sucher and Carles 2008, Vural and Eri 2009), molecular markers specific to *S. chirayita* could be generated and this in turn could yield a molecular diagnostic tool for authentication purpose. Such a diagnostic tool would be valuable for pharmaceutical applications and for pharmacognosy-based research (Joshi et al. 2004) as well as for intellectual property rights protection (Sharma et al. 2009).

Materials and methods

Plant materials For the RAPD PCR optimization, fresh DNA samples were collected from Godawari, Lalitpur; the rest of the DNA samples were collected in silica gel (**Table 1**). Collected samples from various part of the country were brought to the NAST Biotechnology Laboratory for DNA extraction and analysis.

DNA extraction and DNA estimation Two main DNA extraction techniques (Doyle and Doyle 1990, Graham et al. 1994) were assessed for their usefulness in generating DNA profiles of *S. chirayita* using RAPD-PCR. DNA quantification

Table 1. Details of *S. chirayita* collections for the present study

District/Locality	Region in Nepal*	Number of samples	Altitudinal range
Ilam/ Maipokhari to Maimayuwa	E	15	2100–2600 m
Lalitpur/ Phulchowki	C	15	2150 m
Kathmandu/ Nagarjun	C	15	2000 m
Terhathum/ Tirikhimti to Guphapokhari	E	15	1500–2800 m
Dhankuta/ Pakhribas	E	15	1750 m
Sankhuwasabha/ Lampokhari, Shreemane, Manlabre and Chauki	E	15	2600–2950 m
Kaski/ Sikles	W	15	2000–2500 m

* W = Western, C = Central, E = Eastern

as well as quality assessment was carried out using a Biophotometer (Eppendorf – AG 22331, Germany).

Gel electrophoresis The quality of extracted DNA was also assessed using 1.5% agarose gel electrophoresis (in an EMBI TEC Santiago, CA gel tank) in 1X TBE buffer [10X TBE; 108 gm Tris base, 55 gm Boric Acid and 40 mL of (0.5 M) EDTA pH 8.0] at 50 V (8.47 V/cm) for half an hour. PCR amplification products were analyzed at 25 V (4.2 V/cm) for 1.5 h using the same buffer system.

The gels were stained with ethidium bromide (10mg/ml solution) for 45 minutes and de-stained for 15 minutes in water prior to visualization and photography using UV trans-illuminator (UVITEC, Japan) and Polaroid Gelcam (UK).

RAPD-PCR optimization The RAPD-PCR reaction conditions were optimized by varying key parameters ($MgCl_2$, dNTPs, template DNA, primer and *Taq* polymerase concentration; **Table 2**). Selection of the best RAPD cycling conditions was carried out through the assessment of two randomly selected RAPD-PCR cycling conditions (*viz*. Yu and Pauls 1992, Edwards 1998). All PCR reactions were carried out in the final volume of 25 µL.

Results and discussion

We first compared two DNA extraction protocols in some of our samples. The method developed by Graham et al. (1994) produced multiple sharp bands on electrophoresis gels (**Plate 1**). Doyle and Doyle's (1990) method produced fewer bands. Both techniques produced reasonably pure DNA (A260/280 ratio ranged from 1.8–2.0) for PCR amplification. Graham et al.'s technique was used in all subsequent DNA extractions from multiple collections. The Graham et al. extraction buffer is comprised of 2% CTAB, 1.4 M NaCl, 0.1 M EDTA and 0.1 M Tris HCl pH 8.0. Principal components of this DNA extraction buffer are cetyl trimethyl ammonium bromide (CTAB), sodium chloride (NaCl), ethylene diamine tetra acetic acid (EDTA) and Tris-HCl. CTAB may bind to poly-phenolic compounds during extraction by forming a complex with hydrogen bonds and may help in removing impurities to some extent (Padmalatha and Prasad 2006). RNA that can be co-isolated with DNA can chelate Mg^{2+} and reduce the yield of the PCR. In the present investigation, RNAse has been incorporated in the TE Buffer, which was used to re-suspend DNA pellets at the end of DNA extraction procedure.

Optimization of RAPD reaction conditions was obtained by varying parameters and selection of the best concentrations for each of the constituent of PCR. The optimized RAPD-PCR conditions comprised of 3.0–3.5 mM of $MgCl_2$, 12.5 ng of

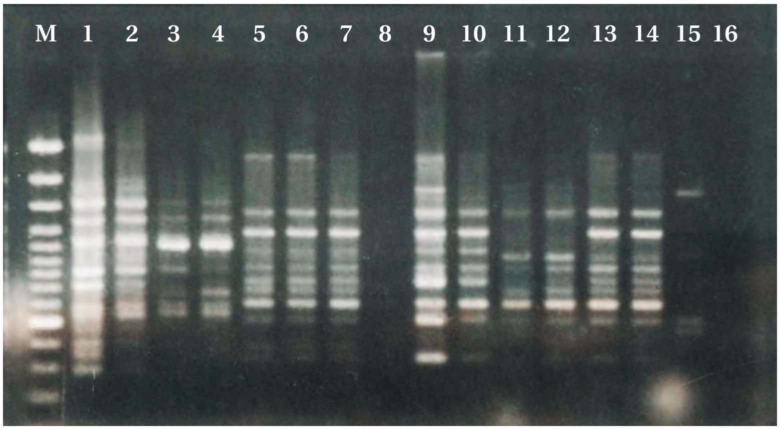

Plate 1. RAPD profile of *S. chirayita* genotypes generated by primer UBC 2 and optimized RAPD reaction parameters. Lane marked M is 100 bp ladder molecular weight marker; lanes 1–2: CTAB extracted DNA (Graham et al. 1994) sample from Godawari, Lalitpur; lanes 3–4: DNA extracted from Godawari, Lalitpur sample using Doyle and Doyle (1990) method; lanes 5–6, 7–8, 9–10, 11–12 and 13–14 are CTAB extracted DNA sample N–1, N–4, N–6, N–8 and N–9 from Pakhribas, Dhankuta respectively. Lane 16 represents negative control.

Table 2. PCR parameters tested and optimized parameters

PCR parameters	Tested range	Optimized conditions found	Remarks
DNA concentration (ng)	12.5, 25, 50, 75, 100, 125, 150, 175	12.5 ng	Highest no. of amplified products observed at 12.5 ng concentration of DNA. Absence of bands from higher concentrations was observed.
$MgCl_2$ concentration (mM)	1.5, 2.0, 2.5, 3.0, 3.5, 4.5, 5.0	3.0–3.5 mM	Lower no. of bands observed at lower (2.0–2.5mM) and higher (4.0–4.5mM) concentrations.
dNTPs concentration (mM)	0.1, 0.2, 0.3, 0.4, 0.5	0.1–0.2 mM	Increased concentration reduced intensity and number of amplified products.
Primer concentration (mM)	0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6	0.2–0.3 mM	Intensity of amplified bands was same at 0.1–0.5mM concentration. Intensity of individual band, number of amplified products and high molecular weight products decreased from 0.6 mM – 1.6 mM concentrations.
Taq polymerase concentration (5U/mL)	0.5, 1.0, 1.5, 2.0, 2.5	2.0U– 2.5U	Faint bands observed at lower concentrations (0.5U–1.5U).

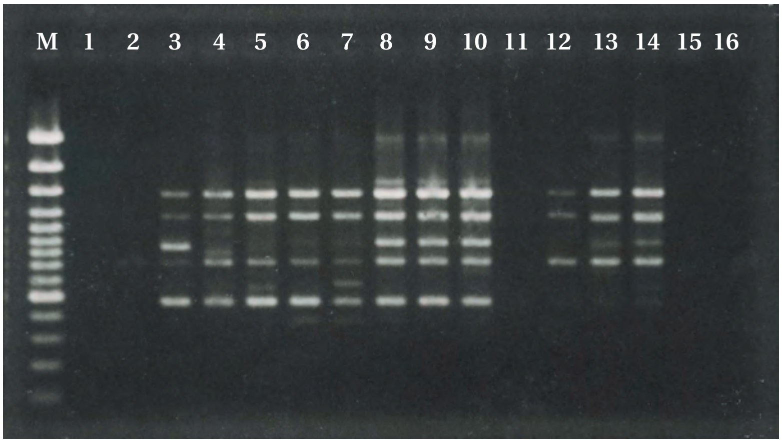

Plate 2. RAPD-PCR optimization for the selection of best $MgCL_2$ concentration for *S. chirayita* using UBC primer 1 and 12.5 ng of DNA. Lane marked M is 100 bp ladder molecular weight marker. Lanes 1–2, 3–4, 5–6, 7–8, 9–10, 11–12, 13–15 represent 1.5 mM, 2.0 mM, 2.5mM, 3.0mM, 3.5mm, 4.0 mM, 4.5 mM concentration of $MgCl_2$, respectively. Lane 16 represents negative control.

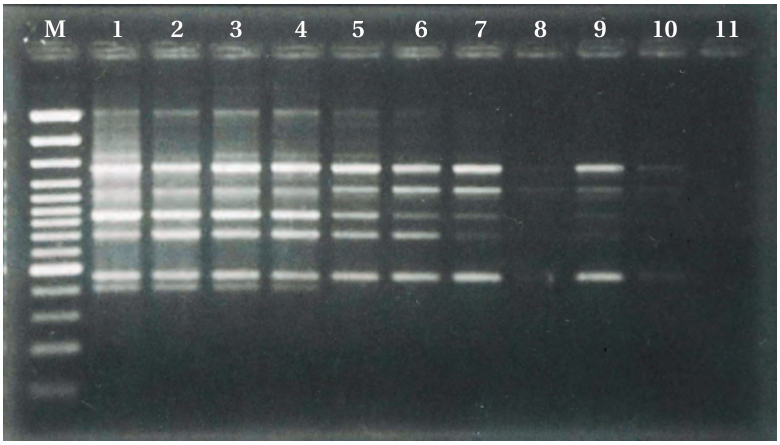

Plate 3. RAPD-PCR optimization for the selection of best dNTPs concentration for *S. chirayita* using UBC primer 1 and 12.5 ng of DNA. Lane marked M is 100 bp ladder molecular weight marker. Lanes 1–2, 3–4, 5–6, 7–8, 9–10 represent 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM and 0.5 mM concentration of dNTPs, respectively. Lane 11 represents negative control.

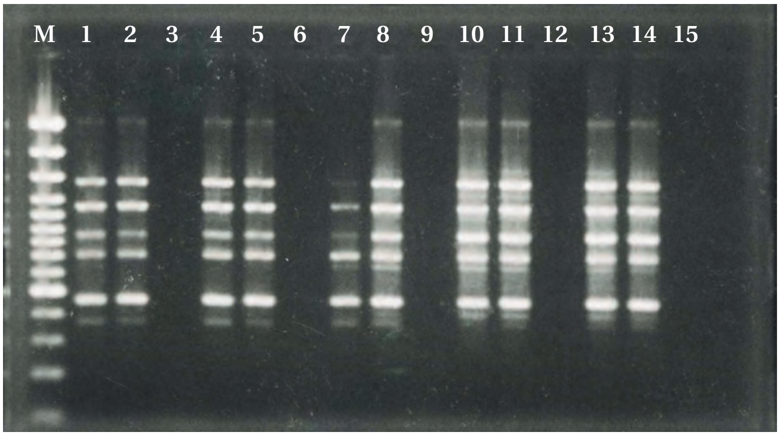

Plate 4. RAPD-PCR optimization for the selection of best *Taq* polymerase concentration for *S. chirayita* using UBC primer 1 and 12.5 ng of DNA. Lane marked M is 100 bp ladder molecular weight marker. Lanes, 1–2, 4–5, 7–8, 10–11, 13–14 represent *Taq* polymerase concentration ranging from 1.0 U, 1.5 U, 2.0 U, 2.5 U and 3.0 U, respectively. Lane 15 represents negative control.

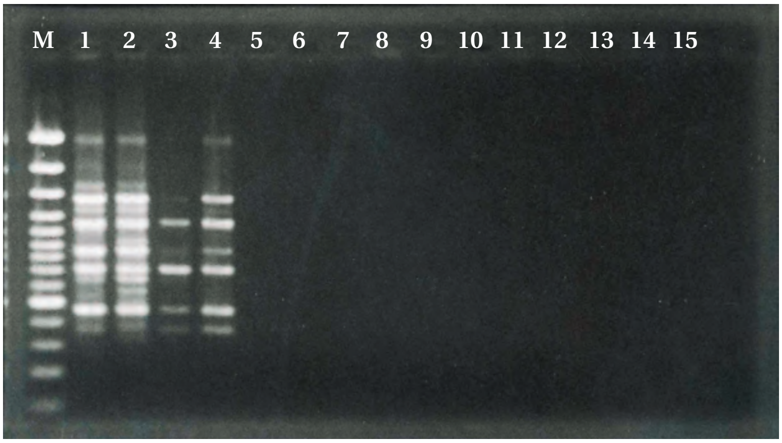

Plate 5. RAPD-PCR optimization for the selection of best DNA concentration for *S. chirayita* using UBC primer 1. Lane marked M is 100 bp ladder molecular weight marker. Lanes 1–2, 3–4, 5–6, 7–8, 9–10, 11–12 represent 12.5ng, 25 ng, 50 ng, 75 ng, 100 ng, 125 ng concentration of template DNA, respectively. Lane 13 represents negative control.

template DNA, 0.1–0.2 mM each of dNTPs, 0.2–0.3 mM each of primers, and 2.0–2.5 units of *Taq* DNA polymerase (MBI Fermentas Company) (**Table 2**). The optimized reaction concentrations were then used in subsequent primer screening and RAPD profiling experiments (**Plates 2, 3, 4 and 5**).

Optimization of reaction parameters in RAPD is crucial in order to maintain reproducibility of RAPD phenotypes among laboratories. In order to generate reproducible RAPD fingerprint profiles, two parameters, *viz.,* quality and quantity of template DNA, have been considered the primary factors affecting reproducibility. Hence controlling these two factors is essential in order to ensure reproducible RAPD patterns (Weeden et al. 1992, Micheli et al. 1997). Apart from DNA, other parameters to be optimized are the concentrations of $MgCl_2$, dNTPs and primers. In the present study, $MgCl_2$ concentration of 3.0–3.5, dNTPs concentration of 0.1–0.2 mM each, primer concentration of 0.2–0.3 mM and *Taq* polymerase concentration of 2.0–2.5 U had no noticeable difference in banding patterns, while template DNA concentration of 12.5 ng was found to be optimal for PCR amplification at all these reagent concentrations.

Of the two randomly selected RAPD-PCR programs (Yu and Pauls 1992, Edwards 1998), the cycling conditions described by Edwards (1998) produced the best profiles for *S. chirayita.* The program consisted of an initial denaturation step at 95°C for 2 min followed by 45 cycles of 95°C for 20 s, 37°C for 60 s and 72°C for 60 s and a final extension step of 72° C for 10 min. The maximum ramp rate available for the PCR machine (Eppendorf, Germany) was used. In some cases, the faster PCR cycle described by Yu and Pauls (1992) would be more applicable. With alfalfa genomic DNA, Yu and Pauls (1992) optimized the denaturing time; they tested 5s, 30s and 60s and found that 5s gave the best PCR products. They also showed that there is a relationship between the time required for primer annealing and the GC content of the primer. For primers having a GC content of 50–80%, a primer annealing time of 30 seconds appeared to be appropriate. It was also shown that strand elongation time affects the size of amplified fragments in the PCR reaction.

For the optimization of RAPD cycling parameters of a number of medicinal and aromatic plants, Padmalatha and Prasad (2006) tested initial denaturation times of 2, 3, 4 and 5 min at 94°C and found 3 min most effective. A range of annealing temperatures (20 to 70° C) and exposure times (30s, 60s, 90s and 120s) was also tested; 37° C for 60 s was found to be the best. In RAPD, random primers should have a minimum of 40% GC content, although 50–80% is generally used (Micheli 1997). While it is generally claimed that RAPD is very sensitive to reaction and cycling parameters, Weeden et al. (1992) concluded that the amplification process is not so sensitive to one or more of the parameters as to seriously affect reproducibility of the technique. Therefore standard reaction conditions and cycling parameters appear to be appropriate for a wide range of plant materials.

Conclusion

Swertia chirayita is one of Nepal's most highly prized medicinal plants both at home and abroad. However, due to the availability of multiple species of *Swertia* in Nepal, not only *S. chirayita* but eight other allied species are also being highly exploited in trade. It is anticipated that the present study aimed at studying genetic diversity within *S. chirayita* populations of Nepal using molecular marker techniques such as RAPDs will furnish valuable information for the sustainable utilization and conservation of these natural resources. Furthermore, DNA samples collected during this investigation and the information thus generated will be highly valuable for planning future molecular projects focusing on *S. chrayita* as well as other *Swertia* species found in Nepal.

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Malaxis biaurita (Lindley) Kuntze (Orchidaceae): a new record for Nepal

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Malaxis biaurita (Lindley) Kuntze (Orchidaceae) has been reported for the first time in Nepal. The species occurs in the subtropical forest at an elevation of 1400 meters. The identifying characters are purple-red flowers, apically entire, obtuse labellum. Detailed description, illustration and relevant notes are provided.

Key words: orchids, taxonomy, distribution

The genus *Malaxis* was established by O Swartze in 1788. The genus comprises about 300 species worldwide, found primarily in tropical and subtropical regions of South East Asia with few species in temperate regions (Pearce and Cribb 2002). *Malaxis biaurita* (Lindley) Kuntze is distributed in the subtropical region of the Andaman Islands (Pradhan 1979). It is also reported in the north-west Himalaya of India (Deva 1982).

Genus *Malaxis* is represented by ten species in Nepal (Rajbhandari and Dahal 2004). *Malaxis biaurita* had not been previously reported from Nepal (Hara et al. 1978; Banerji and Pradhan 1984; Press et al. 2000; DPR 2001; Rajbhandari and Dahal 2004; Rajbhandari and Baral 2010). There is no record of this species in the National Herbarium, Kathmandu (KATH) or Tribhuvan University Central Herbarium (TUCH). Here we report a new record of *Malaxis biuarita* (Lindley) Kuntze for Nepal. The first author collected it in Lamidanda, Makwanpur District, Narayani Zone (Central Nepal) at an altitude of 1400 meter and deposited it at KATH.

Malaxis biaurita (Lindley) Kuntze, Gen. Pl. 2: 673. 1891.
Microstylis biaurita Lindley, Gen. Sp. Orchid. Pl. 20. 1830.
Herb, terrestrial, up to 40 cm tall. **Stem:** fleshy, cylindrical, 2–2.5 cm, enclosed in leaf sheaths. **Leaves:** ascending; petiole sheath-like, 1.5–3 cm, clasping; leaf blade ovate, oblong-ovate, or sub-elliptic, 6–12×1.8–4.5 cm, base contracted into a stalk, apex acute or acuminate. **Scape:** erect, 14–21 cm, wingless; raceme 8–10 cm, 20–30 laxly flowered. **Floral bracts:** reflexed, 4.5–6 mm, narrowly lanceolate, apex acuminate. Pedicel and ovary 4–5 mm. **Flowers:** purplish red, 8 mm across. **Abaxial sepal:** oblong-lanceolate, 5–5.5×1.5–2 mm, both margins revolute, apex obtuse; **lateral sepals:** narrowly oblong-ovate, oblique, 6×1.5–2 mm, apex obtuse. **Petals:** narrowly linear, 5×0.2 mm, apex blunt. **Labellum:** ovate-lanceolate in outline, 7 mm, both ends tapering, base with two falcate auricles, apex obtuse. **Column:** 1 mm thick.

Flowering: July.

Figure 1. *Malaxis biaurita* habit

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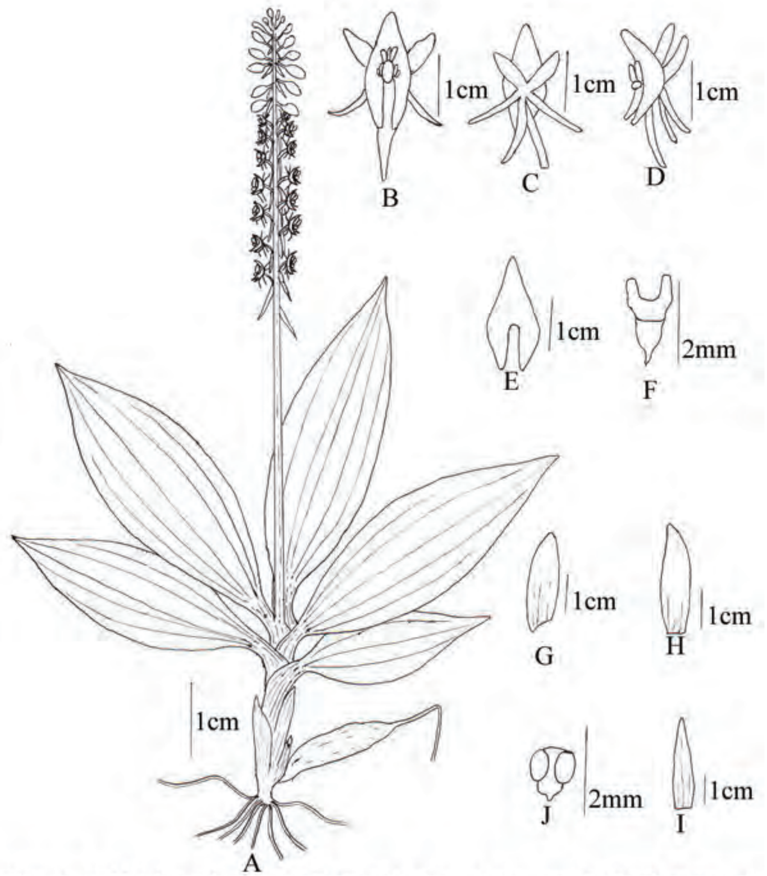

Habitat: Cool growing terrestrial on humus rich sandy slopes, likes partial shade, occurs in the subtropical forest margins at an altitude of 1400 meters.

Occurrence: Central Nepal, Narayani Zone, Makwanpur District, Lamidanda, 25 July 2007, Raskoti 204 (KATH).

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Figure 2 (top on the left). A. Habit of the plant; B. Front view of flower; C. Back view of flower; D. Side view of flower; E. Labellum; F. Column; G. Dorsal sepal; H. Lateral sepal; I. Petal; J. Anther. Sections of the figure (A to J) are not in the same scale.

Figure 3 (left). Close up view of the flower with sepals, petals, lip and column visible.