Detection Survey Protocol for Cowpea Weevil, *Callosobruchus maculatus* (Fabricius) in Medicinal and Aromatic Plants Nepal

Government of Nepal
Ministry of Agriculture and Livestock Development
Plant Quarantine and Pesticide Management Centre
Hariharbhawan, Lalitpur

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NPPO-Nepal, 2025

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1. Introduction

Callosobruchus maculatus (Fabricius, 1775) is one of the most damaging pests of stored grain legumes (Seram et al., 2022). It is also found in the mature pods in the field. This pest is native to Africa but is distributed globally including different countries of Asia, Africa, Europe, America and Australia (Maslov et al., 2006). It is found in warm humid conditions of tropical, subtropical and warm temperate regions. *C. maculatus* a major pest of cowpea, lentil and green gram but it also attacks other crop species such as pigeon pea, cashew nut, soybean, and chickpea. Adult female weevil lays eggs on the surface of the host seed, the larvae penetrate the seed, feeds internally and causes significant damage. Infested seeds lose weight, quality, and viability, which can severely impact stored food grains (CABI, 2022).

China has listed *C. maculatus* as a quarantine pest while exporting medicinal plants from Nepal. The agreement signed between General Administration of Customs of the People's Republic of China (GACC) and Government of Nepal (GoN) has provision of ensuring the medicinal plants to be exported from Nepal should be free from this pest. Plant Quarantine and Pesticide Management Centre is authorized by the government of Nepal as NPPO, and under Plant Protection Act 2064, Clause 6 (2), survey and surveillance function and responsibility is designated to NPPO as per the sub clause (i) "To perform such other function as prescribed"

For the continuous and effective trade of medicinal plants between Nepal and China, the detection survey for the presence of this pest has to be carried in regions where the plants are being produced, stored or processed while, it could be of serious concern in already infested storage where the medicinal plant products are being stored. Because, proper pest detection and pest identification are crucial for the appropriate application of phytosanitary measures (ISPM-4 (Requirements for the establishment of pest free areas), ISPM-6 (Guidelines for surveillance), ISPM-7 (Phytosanitary certification system), ISPM-9 (Guidelines for pest eradication programs) and ISPM- 20 (Guidelines for a phytosanitary import regulatory system) (FAO 2020). This survey protocol may guide the surveyors, government officials in the quarantine check-post and others for conducting the detection survey and identifying the pest successfully.

This detection survey protocol is developed to establish standardized procedures for detecting and monitoring *C. maculatus* in field and storage facility of MAPs to be exported to China. This will help in conducting the detection survey and surveillance of *C. maculatus* within the national territory. It is also expected that the protocol will facilitate in evidence-based decision making for phytosanitary measures.

2. Taxonomic tree

Domain: Eukaryota
Kingdom: Animalia
Phylum: Arthropoda
Subphylum: Hexapoda
Class: Insecta
Order: Coleoptera
Family: Bruchidae
Genus: *Callosobruchus*
Species: *Callosobruchus maculatus* (Fabricius, 1775)

Source: (CABI, 2022)

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3. Biology/life cycle

. *maculatus* is a holometabolous insect which passes through egg, larva, pupa and adult stages in its life cycle. The duration of life cycle depends on temperature, relative, humidity and host species. After 24-48 hour it exits from the host seeds, adult becomes mature for sexual reproduction. A female beetle lays more than 100 eggs during its lifetime singly on the seed surface (Garima et al., 2022). The optimum temperature for oviposition is 30-35°C. The detritus produced after hatching of insect egg makes it clearly visible to naked eye. The larva bore hole inside the seed, and reaches to cotyledons as it grows. There are four instars in its larval phase (CABI, 2022). The first instar duration is 6 to 7 days, second instar lasts 3-4 days and third larval stage is completed within 3 to 4 days at 28.05±2 °C, 71.07±3 % RH. The third larval stage is very active, rapidly consumes the seed endosperm and increases in size to attain its full form. The larval duration lasts for about 15 to 19 days. The entire larval stage completes inside the seed. After final molting, it undergoes pupal stage. The pupal duration is of 4-5 days (Salunkhe & Gaikwa, 2023). The adult beetles have two morphological forms: sedentary and winged form. Sedentary forms lay higher number of eggs and winged form can undergo dispersal rapidly. The adult is short lived (about 15 days under favorable conditions) and do not feed on stored products (CABI, 2022). Both sexes are polygamous in nature and the females that mate with multiple virgin males live longer, lay more eggs, and produce larger eggs (Garima et al., 2021). The optimum environmental condition for growth and development of this is about 32°C and 90% RH. *C. maculatus* attacks the host in field and eventually passes to the storage conditions (CABI, 2022).

Egg: The eggs are glued to the surface of pulses or other suitable hosts. They are smooth, domed structures with oval flat bases (CABI, 2022). The egg is 0.7mm long and 0.4mm in width (Maslov et al., 2006). The egg can be seen with naked eyes under careful observation or can be observed under stereomicroscope.

Larva: The creamish to whitish larvae is normally only found in cells bored within the seeds of pulses (CABI, 2022). The larva is coiled (C-shaped), hairless, and legless. It measures up to 4mm in length (Maslov et al., 2006).

Pupa: The pupa is whitish. It is about 3.87 mm in length and 1.76 mm in width. When the larva starts to pupate, the outer shell becomes thinner (Garima et al., 2021).

Adult: The body of adult is about 3 mm in length and 1.7 mm in breadth. Typically, adults with reddish brown body have black patches on the outer edge of the elytra. The lighter portion of the elytra is covered with greyish yellow hairs, together forms 'H' shaped pattern (Maslov et al., 2006). Different species of *Callosobruchus* can only be identified distinctly through their adult stages, especially with the help of typical characteristics of antennae, hind femur and aedeagus. The *C. maculatus* antennae are slightly serrated in both male and female (Seram et al., 2022). The pronotum lacks lateral carinae (ridge). The basal tubercle may be present or absent on the elytra. Both the external and internal carinae of the hind femur have a sub-apical spine or denticle. The outer denticle in the expanded carinate of straight hind tibia is shorter or equal in length than the inner denticle. Aedeagus is the most important organ for the identification of *C. maculatus*. Aedeagus or male genital organ has triangular ventral valve, an internal sac, and shorter median lobe. The lateral lobes of the aedeagus are deeply split, flattened, and unaltered at the apex (Seram et al., 2022).

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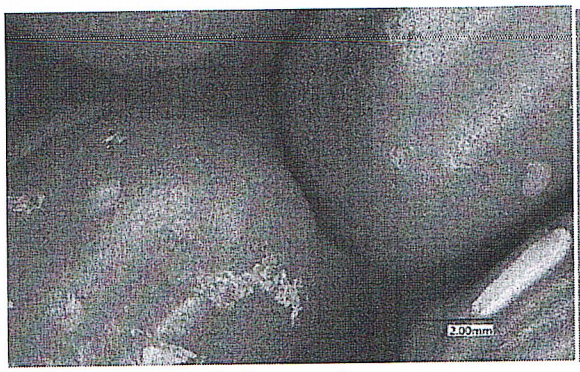
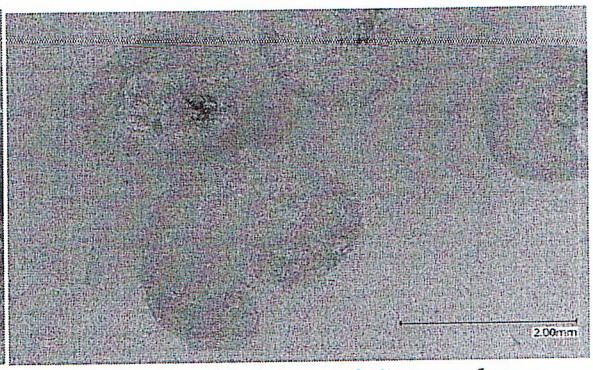
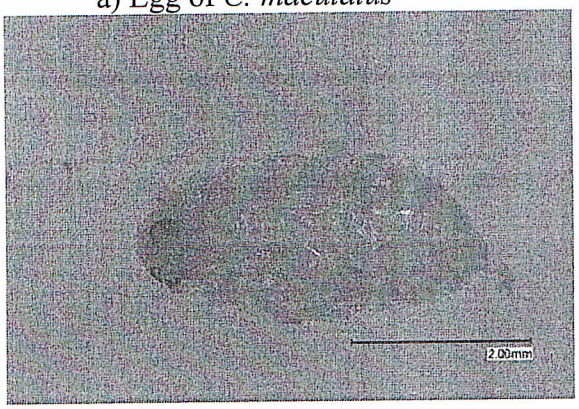


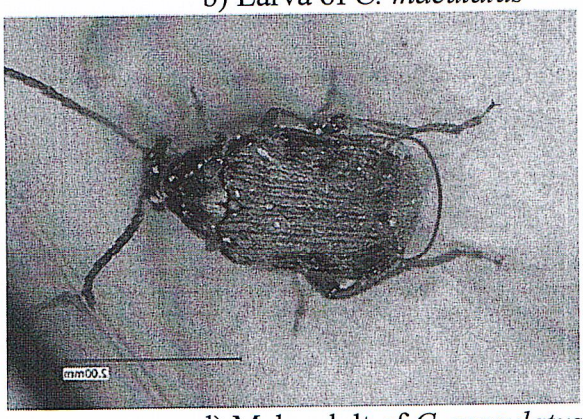
a) Egg of *C. maculatus*



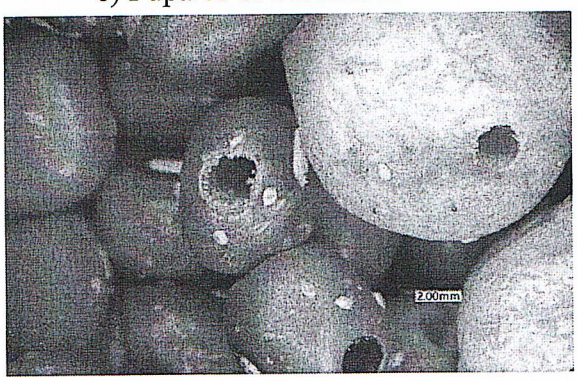
b) Larva of *C. maculatus*



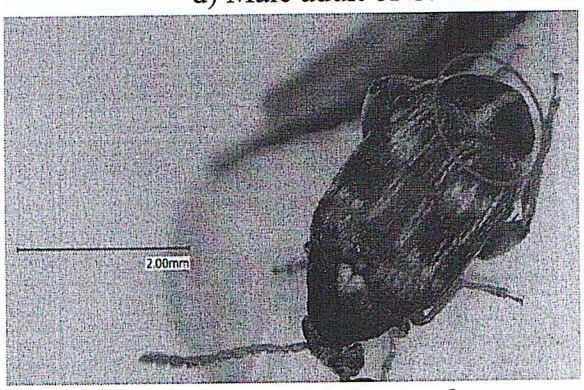
c) Pupa of *C. maculatus*



d) Male adult of *C. maculatus*



e) Typical damage symptom



f) Female adult of *C. maculatus*

Fig. 1: Different stages of *C. maculatus*

Source: (Garima et al., 2021)

4. Mode of dispersal

The adult stages have flying forms which can fly up to 3-5 km distances (Maslov et al., 2006). Hence, for short distance movement, flight is used by this species. While, it is transported long distances through internal and international trade of infected seeds, contaminated containers, storage materials and other agriculture equipment.

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6. Host range

C. maculatus attacks wide range of hosts. There are some major hosts and other minor hosts. The host range of *C. maculatus* described by CABI (2022) is given below:

- Fabaceae: Chickpea (*Cicer arietinum*), soybean (*Glycine max*), cowpea (*Vigna unguiculata*), mung bean (*Vigna radiata*), black gram (*Vigna mungo*), pigeon pea (*Cajanus cajan*), groundnut or peanut (*Arachis hypogaea*), pea (*Pisum sativum*), lentil (*Lens culinaris*) and adzuki bean (*Vigna angularis*),
- Arecaceae: *Cocos nucifera* (coconut).
- Malvaceae: *Gossypium hirsutum* (cotton)
- Asteraceae: *Helianthus annuus* (sunflower)
- Pedaliaceae: *Sesamum indicum* (sesame)

However, the concerned medicinal plants have not been detected as host of *C. maculatus* yet, while it is the quarantine pest of following hosts as per the China trade list.

S N	Scientific Name	S N	Scientific Name
1	*Phyllanthus emblica*	9	*Polygonatum kingianum* *Polygonatum sibiricum* *Polygonatum cyrtonema* *Polygonatum cirrhifolium* *Polygonatum verticillatum*
2	*Paris polyphylla*	10	*Amomum subulatum*
3	*Aquilaria sinensis* *Aquilaria malaccensis* *Aquilaria agallocha*	11	*Ganoderma lucidum* *Ganoderma sinense*
4	*Herpetospermum pedunculosum*	12	*Rubia wallichiana* *Rubia tibetica* *Rubia spp.*
5	*Murraya exotica* *Murraya paniculata* *Murraya koenigii*	13	*Piper longum*
6	*Cassia obtusifolia* *Cassia tora* *Senna tora*	14	*Ferula sinkiangensis* *Ferula fukanensis* *Ferula narthrex*
7	*Santalum album*	15	*Justicia adhatoda*
8	*Swertia chirayita*		

7. Detection survey

The basic procedure is to perform detection survey; the General Surveillance is done to know whether the insect is present in the country or not. In case of its presence, the detection survey is conducted with the methods described below:

7.1 Visual inspection method

In the visual inspection method, the warehouse, equipment used in the storage, bags, surroundings, cracks and crevices around the storage structure should be carefully inspected. Careful search, observation and accurate recording of results must be practiced following reliable method. The method relies on the knowledge and experience of the surveyor. During carrying out inspection,

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some of the randomly chosen bags should be sampled, as well as bags corner, surface, folds should be examined. Some of the bags should be lifted and agitated to trace the infestations (Semple et al., 1992). Some of the hosts might have kept closer to the medicinal plants that might be the cause of infestation. In such case, the infestation in the nearby storage of kidney bean or common bean or any other host plants should be examined. The packaging and processing areas of medicinal plants should also be included for inspection.

7.2 Trapping method

Traps offer a potential approach for estimating the degree of infestation and can also be used to detect light infestations (Semple et al., 1992). There are different types of traps that can be used for the detection of *C. maculatus,* such as probe trap, pitfall trap, pheromone trap, phytochemical based attraction lure, etc. Appropriate trap can be set depending on the situation i.e. in the field and stored conditions. For example, Probe type trap is a method that can be used in storage bags to determine the presence of the pest. In this method, the probe is inserted inside the store bags and left for three hours. The *C. maculatus* adult crawling inside the bag can be collected with this method. The pitfall trap is used in the field for monitoring the pest using general type of pitfall trap. Depending on the availability on the market, The 3-methyleneheptanoic acid, (Z)-3-methyl-3-heptenoic acid, (E)-3-methyl-3-heptenoic acid, (Z)-3-methyl-2-heptenoic acid, and (E)-3-methyl-2-heptenoic acid can be used as a synthetic female sex pheromone. This can be used individually or in combination to attract *the* male adults (Philips et al., 1996). However, the sex pheromones of *C. maculatus* are not yet available commercially (CABI, 2022). The adults are attracted towards the host for oviposition through their unique host detection mechanisms (CABI, 2022). In the storage, the adults can be attracted using the most preferred host of *C. maculatus* such as cowpea (*Vigna unguiculata*), Lentil (*Lens culinaris*), chickpea (*Cicer arietinum*) and green gram (*Vigna radiata*). About 100 grams of legume grains should be placed on the clean dry container. Monitoring can be done every 2 to 3 days. The seed should be replaced every week for increased efficacy.

Black light traps can be used effectively in warehouse to capture *C. maculatus* adults (Gilbert & Baur, 1984; Kalpna et al., 2022). It is non-specific to species and may trap diverse insect species. A 32 Watts black light trap should be mounted in the light trap set-up with rain shed and the collection container. Normal 25 W incandescent bulb can also be used for trapping *C. maculatus* but it is less effective than black light in case of this pest (Keever & Cline, 1983).

8. Habitat

Most favorable situation for oviposition and growth is the tropical climate but it is also present in the temperate climatic conditions where there is favorable environments such as availability of hosts, suitable temperature and relative humidity, as well as in the storage (CABI, 2022).

9. Reports in Nepal

It has been reported in various regions of Nepal and has been studied by many scientists (Khanal et al., 2020; Neupane et al., 2016; Paneru & Siwakoti, 2000; Subedi, 2015) under diffetent topics after this pest became evident in various regions of Nepal. Therefore, it is well known and widespread pest of Nepal.

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10. Purpose

- To detect *C. maculatus* from the randomly selected medicinal plants in production areas (sample to be taken in areas from where the medicinal plant products are collected).
- To support NPPO to declare pest free area.
- To report to organization such as IPPC, GACC etc. for the facilitation of trade of medicinal plants.

11. Scope

The survey will cover the randomly selected storage and field locations. The host location specific details will be collected from various sources like Department of Plant Resources, Department of Forest and soil conservation, National Herbarium and Plant Laboratories, and other related institutions like NARC Research Stations, Central Department of Botany of Tribhuvan University, Kathmandu University, Agriculture and Forestry University, NGO, INGOs and other published materials.

12. Target pest

Preferred Scientific Name: *Callosobruchus maculatus* (Fabricius, 1775)
Preferred Common Name: Cowpea weevil
English common names:

- Cowpea seed beetle
- Four-spotted bean weevil
- Southern cowpea weevil
- Spotted cowpea bruchid

13. Timing of survey

Warm season is the most suitable season to conduct the survey of this pest. It is mostly prevalent from June to September. During this period, *C. maculatus* have optimum environmental conditions such as temperature, relative humidity and availability of hosts for its growth and development.

Table 1. Sampling schedule for detection survey of *C maculatus* across various sites

Production site	Sampling frequency			
	June	July	August	September
Site 1	June (1st week)	July (1st week)	August (1st week)	September (1st week)
Site 2	June (2nd week)	July (2nd week)	August (2nd week)	September (2nd week)
Site 3	June (3rd week)	July (3rd week)	August (3rd week)	September (3rd week)
Site 4	June (4th week)	July (4th week)	August (4th week)	September (4th week)

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14. Location of survey

Survey will be conducted in local storage house, warehouses, packaging and processing areas of medicinal and aromatic plants products. The field (wild or cultivated), where the medicinal plants are produced or collected will also be included in the survey.

15. Design of survey program

15.1 Sampling method

C. maculatus is the common storage pest and mostly found in the storage areas. For the pest detection, storage area should be checked carefully, i.e. entry, exit and surrounding areas. The possible pathway of occurrence of this pest in the storage should be observed for example: the movement of products, containers, or people handling such products, which might get exposed to this beetle should be checked. Storage structures such as local storage equipment, sacks, woodwork, loose plaster, loose paint, cracks and other potential hiding spots should be observed. The proximity of nearby bean storage should also be observed (NPPO, 2022).

For sampling in storage area, random number should be assigned to the sampling units such as the stored bags with the random sampling applied for selection and inspection for the detection of the pest. While surveying in the field, adequate, representative samples should be collected that will support in accurately detecting the pest. Diagonal methods are used for quickly scanning the presence of pest. However, more representative samples are obtained from W diagonal and random sampling method. For detection survey, W method or random sampling design can be used.

15.2 Sample size

Total sample size will be calculated based on the following formula:

$$\text{Sample size} = \frac{-\log(1 - \text{confidence level})}{\log(1 - \text{Design prevalence}}$$

The design prevalence for detection survey is 1% (NPPO-Nepal, 2024). Hence, from the Table 2, minimum 298 samples should be taken for detecting the pest at 95% confidence level.

Table 2. Calculated sample size for different design prevalence at different confidence level

Confidence Level	1 – Confidence Level	Design Prevalence	1 – Design Prevalence	Sample Size
0.95	0.05	0.01	0.99	298
0.95	0.05	0.02	0.98	148
0.99	0.01	0.01	0.99	458
0.99	0.01	0.02	0.98	228
0.95	0.05	0.001	0.999	2,994
0.95	0.05	0.002	0.998	1,496
0.99	0.01	0.001	0.999	4,603
0.99	0.01	0.002	0.998	2,300

Number of samples per sampling point differs according to the medicinal plant species to be observed. Table 3 gives the criteria for selecting the minimum number of samples per sampling point.

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Table 3. Minimum number of samples to be obtained from each sampling point

Medicinal plants	Minimum number of samples
Herbs species *(Swertia chirayita, Polygonatum* spp*)*	5 plants/ sampling point
Specimen associated with tree *(Ganoderma lucidum)*	2 tree per sampling point and 2 mushroom/ tree
Tree species (*Phyllantha emblica, Piper longum)*	2 tree/ sampling point and 10 fruits per tree

16. Materials required

The listed equipment are essential for conducting survey in storage area.

- Containers with ventilation (for live insect)
- Collection jar
- Magnifying lens
- Camel hair brush
- Ziplock bag
- Data-sheet
- Magnifying lens
- Diagnostic keys
- Envelops
- Alcohol/water resistant pen
- Labels
- Killing jar
- Absolute alcohol
- GPS measuring tool
- Camera/ mobile phone
- Markers
- Tweezers
- Traps/lures
- Collector tags
- Cotton rolls
- Ethyl acetate
- Glass vials
- Data sheets
- Zip-lock bag

The listed equipment are needed for the field survey.

- Mosquito repellent
- GPS measuring tool or Geometer
- First-aid kit
- Permits
- Camera or mobile phone
- Data-sheets
- Field guide
- Water-proof/alcohol-proof pens

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- Labels
- Zip-lock bag
- Paper bags
- Note-book
- Magnifying lens
- Insect collection tubes
- Absolute alcohol and 70-90% alcohol
- Forceps
- Ethyl acetate
- Cotton rolls
- Identification keys of the specimen
- Measuring tape
- Aspirator
- Traps

Source: (NPPO-Nepal, 2024)

17. Collection and preservation of specimen

17.1 Collection from storage

- Adult, larva and pupal stages of insects should be collected with sterilized camel hair brush or by using an aspirator.
- Specimens should be placed in well ventilated container for safe shipping if the identification is not done immediately at the same locality.
- For convenience of handling, the adult in the container can be kept in the freezing temperature for 2 hours (NPPO-Nepal, 2024).
- For collecting larva and pupa, the seed sample should also be collected. If found sample seeds with visible eggs on the surface of the seed, such samples should be collected and handled carefully to avoid the damage to the eggs.
- Multiple samples are always better.

17.2 Collection from field

According to NPPO-Nepal (2024), following things need to be considered while collecting the specimen from the field.

- Before collecting, the equipment needed for collection should be sterilized with 70% ethanol or 0.5% chlorine solution.
- The specimen should be collected and handled very carefully as it may damage its diagnostic feature for identification due to carelessness.
- All the life stages of specimen should be collected whenever possible.
- The collected specimens must have intact appendages like antennae, wings, and legs.
- The adult weevil should be folded in tissue paper to protect its appendages. Well ventilated container is most suitable for transportation.
- If possible the sample should be stored in a secure, cool, and dark place. The stored sample can be kept in a freezer for 2 hours before dispatch to kill the insect for the convenience of handling.
- All the samples should be well-labelled.

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17.3 Preservation of specimen

The most common technique for preserving immature insects is to collect them and store them in a vial filled with 70% ethanol (alcohol). Boiling in water before preservation can be used to maintain color in soft bodied insects and then placed in 65% ethyl alcohol and the container should be completely filled to prevent its movement and damage (NPPO-Nepal, 2024). Immature stages are preserved in fluid (stored in 85–90% ethanol, preferably after fixation in KAA or Carnoy's fluid).

17.4 Labeling the specimen

The collected weevil should be labelled with the help of alcohol or water resistant inks. The labelling should be done both inside and outside the jar. While labelling the specimen following this need to be considered in the label (NPPO-Nepal, 2024):

- Host name (Scientific and common name)
- Host commodity Plant parts affected by the pest
- Pest's scientific name and life stage
- Family or order of the pest
- Location details
- Collection date
- Name of collector

18. Morphological diagnosis

For successful identification of *C. maculatus*, adult specimen is used. The method of preparation of adult for identification of weevil as described by Seram et al. (2022) is described below.

I. Well preserved specimen with all the appendages and organs should be selected for dissection and identification of the species.
II. The selected specimen and equipment should be well sterilized in 70% ethanol or 0.1% Sodium hypochlorite solution for one minute.
III. The sterilized adults should be kept in separate vials with 10% potassium hydroxide solution. This helps in softening of tissues and dissolution of fat.
IV. The abdominal parts containing the genital organs should be dissected which can be done with the help of stereomicroscope.
V. The aedeagus of male weevil should be carefully dissected. For this operation, microneedle and forceps can be used. Special consideration should be given while carrying out this step to avoid damage to the organs.
VI. The aedeagus should be cleaned and stained with the help of staining agent (Fuchsin acid dissolved in acetic acid).
VII. For improved transparency, the stained parts should be transferred to the clearing solution (2:3 carboxylic acid + xylene solution).
VIII. Now, the specimen should be mounted on the observing glass slides using Canada balsam. Glycerol can also be used as a mounting agent for preparing temporary slide.
IX. The female genitalia (8th and 9th segment), legs, forewings, antennae should also be prepared in a permanent or temporary slide following the step I to step VIII.

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19. Molecular diagnosis

Molecular diagnosis of *C. maculatus* can be done by several methods such as real time Polymerase Chain Reaction (RT-qPCR), DNA barcoding and DNA sequencing. For carrying out PCR for diagnosis of *A C. maculatus*, the universal primer can be used (Krishnega et al., 2021). For the molecular diagnosis, the well-preserved specimen are sent to the molecular laboratories of Nepal Agriculture Research Council (NARC), Central Agriculture Laboratory, Universities (AFU, TU) and other accredited private laboratories.

20. Sample analysis and reporting

Surveyor should keep at least one specimen with himself and at least one specimen should be sent to NPPO for identification and future reference. The laboratory should send the report to the NPPO if it has examined and identified the specimen. They should notify the NPPO for the reporting/declaration of insect-pest if the specimen is examined and identified by the Central Agricultural Laboratory, the National Entomology Research Center, or any other organization. The specimen shall be preserved and all records should be securely stored by NPPO (NPPO-Nepal, 2024).

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Annexes

Annex 1. Calendar of activities to follow while conducting detection survey

Phase	Time of the year	Activities
Pre-survey preparations	February-March	Literature review Thoroughly understanding the protocol Training of the surveyors Purchasing the materials required for survey. Co-ordination with the traders, farmers/collectors. Budget allocation for survey
Field survey	May	1st replication of survey
	June	2nd replication of survey
	July	3rd replication of survey
	August	4th replication of survey
Laboratory diagnostics	September-October	Submitting the samples to laboratory for morphological and molecular analysis
Analysis/ reporting	November-December	Preparation of Survey report Submission to NPPO-Nepal Conducting Validation workshop.

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Annex 2. Location-wise monitoring and observation
Medicinal plant producing districts where there is potential of *C. maculatus* detection are listed below:

Region	**Districts**
Mountain	Solukhumbu, Taplejung, Mustang, Mugu, Dolpa
Hill	Dhankuta, Ilam, Panchthar, Terhathum, Sankhuwasabha, Okhaldhunga, Bhojpur, Khotang, Udaypur, Dolakha, Ramechhap, Sindhupalchok, Dhading, Makwanpur, Tanahun, Syangja, Gorkha, Lamjung, Palpa, Gulmi, Myagdi, Baglung, Rukum, Dailekh, Dadeldhura, Bajhang

Proposed districts to be carrying out detection survey of *C. maculatus* in 2081/82

Taplejung
Mugu
Khotang
Terhathum
Sindhupalchok
Tanahu
Gorkha
Bajhang

Annex 3. Details to be recorded in the storage while surveying

Title of the study/survey	
Name of field/site visited	
Date/time of field visit when the pest was intercepted	
Name and the address of local people involved	
Contact details of local people/s involved in the survey	Phone: Email:
GPS reference point	Latitude:
	Longitude:
	Altitude:
Locality	Village name ward no.:
	Local level:
	District:
Climate data of locality	Average min. temp (in °C):
	Average max. temp (in °C):
	Rainfall (in mm)

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Annex 4. Data to be recorded during the survey in field

Date: Host:

Sample number	Infestation Level (Low, medium and high)	No. of Insects Observed	Damage Symptoms	Stage of insect observed	Remarks

Annex 5. Data to be recorded while carrying out survey in store

Date:

Host commodity:

Trap type	Trap density	Location of trap	Insect captured/ trap	Life stage captured	Remarks

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